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(54) Title: COMPOUNDS AND CONSTRUCTS FOR PRODUCING MALE STERILE PLANTS

(57) Abstract

A method is described for inducing male sterility in plants which are capable of being genetically transformed. The plant is tranformed with a DNA-construct that combines a male-organ specific promoter with an enzyme which reacts with a protoxin to release, in the male-organ or gamete, a toxin for the male-organ or gamete. Also described is the DNA-construct, the protoxin, the transgenic plant containing the construct, seeds produced by the transgenic plant, a method for producing hybrid seed, seed progeny, and transgenic plants contacted with the protoxin.

TITLE

COMPOUNDS AND CONSTRUCTS
FOR PRODUCING MALE STERILE PLANTS
BACKGROUND OF THE INVENTION

Hybrid seed production is an important means of introducing desirable traits into agronomically valuable crop plants. For instance, quality traits such as oil content, herbicide resistance, disease resistance, adaptability to environmental conditions, and the like, can be hybridized in offspring so that the latter are invested with the most desirable traits of its parents. In addition, progeny from a cross may possess new qualities resulting from the combination of the two parental types, such as yield enhancement resulting from the phenomenon known as heterosis. Controlled cross-fertilization to produce hybrid seeds has been difficult to achieve commercially due to competing self-fertilization, which occurs in most crop plants.

Currently, hybrid seed production is performed by one of the following means: a) mechanically removing or covering the male organs or gametes to prevent self-fertilization followed by exposing the male-disabled plants to plants with fertile male organs that contain the trait(s) desired for crossing; b) growing genetically male-sterile plants in the presence of plants with fertile male organs that contain the trait that is desired for crossing; or c) treating plants with chemical hybridizing agents (CHA) that selectively sterilize male organs followed by exposing the male-disabled plants to plants with fertile male organs that contain the trait that is desired for crossing.

Some disadvantages to each of these methods include: a) this is only practical for a few crops, such as corn, where the male and female organs are

structurally far apart; and it is labor intensive and
costly; b) genetically male sterile lines are cumbersome
to maintain, requiring crosses with restorer lines; c)
all CHAs exhibit some degree of general phytotoxicity
5   and female fertility reduction. Also, CHAs often show
different degrees of effectiveness toward different crop
species, or even toward different varieties within the
same species. The method of this invention is
applicable to a wide range of crops and allows selfing
10   to maintain lines.

This invention concerns, inter alia, a method for
selectively sterilizing male organs in one parent and
exposing the resulting plant to plants with fertile male
organs to produce seed having the desirable
15   characteristics of both parents and potentially
additional characteristics resulting from the
combination. Plants are rendered receptive to male
sterility induction by introducing certain DNA
constructs. Some background teachings concerning DNA
20   constructs employed herein or related to plant male
sterility are as follows.

EPA 89-329308 discloses constructs containing
antisense DNA and other genes in tobacco, tomato and
Brassica with the prediction that expression of the
25   antisense RNA and toxic proteins will result in male
sterility. Pollen-specific promoters and chemically
regulated promoters are suggested. Also disclosed is
the introduction of β-glucuronidase (GUS) into the
transgenic plant as a marker gene but without
30   recognition that GUS itself has utility in producing
male sterility when the plant is exposed to glucuronide
conjugates of toxins.

A note by Jefferson in Nature 342:838 (1989)
describes that cytotoxic aglycones can be conjugated and

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used to produc tissue-specific toxicity to GUS-
expressing plant cells.

GB 2197653A discloses the production of transgenic
plants containing GUS and the use of constructs to
5   produce said plants. GUS is said to have utility as a
reporter gene for promoter analysis and possibly as a
means to select mutations in fused promoters.

EPA 89-344029 discloses the production of toxic
proteins in the male organs of plants to produce male
10   sterility. Also disclosed is the expression of GUS as a
marker gene in male organs but without the recognition
that GUS itself has utility in producing male sterility
when the plant is exposed to glucuronide conjugates of
toxins as disclosed herein.

15   An article by Goldberg in _Science_ 240:1460-1467
(1988), discloses the anther-specific promoter from the
tobacco TA29 gene. See also EPA 89-344029. This
promoter is useful as a component of constructs employed
in the instant method for producing male sterility in
20   plants.

WO 90/08828 discloses that GUS cleaves a
glyphosate-glucuronide protoxin into a glyphosate toxin.
No teaching is given for the synthesis of glyphosate-
glucuronide, no protoxin is synthesized, no
25   demonstration is provided that glyphosate-glucuronide is
a suitable substrate for GUS or that glyphosate-
glucuronide is nontoxic in plants and there is no
disclosure of sulfonylurea or maleic hydrazide toxins.

Each of the following publications describes
30   representative sulfonylureas that can be conjugated to
saccharides such as glucuronic acid according to the
teachings herein and employed in the disclosed method
for effecting male sterility in plants:

U.S. 4,394,506   U.S. 4,394,506
35   U.S. 4,481,029   EPA 87,780

EP 95,925　　　　　　　　　EPA 161,211
U.S. 4,435,206　　　　　　　Japan 63/166,803A
U.S. 4,522,645　　　　　　　U.S. 4,684,395
U.S. 4,420,325　　　　　　　EP 87,780A.

5

SUMMARY OF THE INVENTION

This invention concerns a method for inducing male
sterility in economically important plants which are, or
can be made, capable of genetic transformation. A plant
10　of choice is transformed with a gene containing the
coding region of an exogenous enzyme and a suitable male
organ-specific promoter. The resulting transgenic plant
produces the exogenous enzyme only in its male organ(s).
Such transgenic plants are male-fertile when grown
15　normally. Unlike normal plants, however, the plants of
the invention can be rendered male-sterile by exposure
to a protoxin that is converted to an active toxin by
the exogenous enzyme in the male organ. The male
sterility trait is only expressed when wanted, by
20　contacting the plant with a selected protoxin; otherwise
the transgenic plant behaves normally.

This "silent" male sterility characteristic is
advantageous in many aspects of hybrid seed production.
Since the protein product coded by the engineered gene
25　is an exogenous enzyme (preferably, GUS) which by itself
does not cause male sterility in the host plant, the
gene can be easily carried in any sexual propagation or
hybridization schemes. The male sterility trait is only
"switched on", at a desired time, by contacting the
30　plant with a protoxin. When such a transgenic plant is
chosen as the female parent for large scale hybrid seed
production, large quantities of its seed can be produced
by selfing. An advantage of this invention is that the
gene coding for the exogenous enzyme in the male organ
35　can be introduced into any desirable breeding lines.

The line harboring the gene can be used either as the
female or male parent.

More particularly, this invention pertains to an
improved method for inducing male sterility in a plant
5   having a male organ comprising the steps:

(i) transforming the plant with a DNA
construct that combines a male-organ specific promoter
with the coding region for β-glucuronidase; and

(ii) contacting the transformed plant with a
10   glucuronic acid-containing protoxin to release a toxin
which renders the male organ sterile;
wherein the improvement comprises:

(a)   employing, in step (i), a promoter
selected from the group TA29$_{1500}$, TA29$_{500}$ (SEQ ID NO:1),
15   p73 (SEQ ID NO:2), p112 (SEQ ID NO:3), p54 (SEQ ID
NO:4), p42W (SEQ ID NO:5), p42 (SEQ ID NO:6) and p158
(SEQ ID NO:7); and

(b)   employing, in step (ii), a protoxin
that comprises a toxin conjugated through a non-acyl,
20   non-phosphoryl hydroxyl residue to glucuronic acid.

Preferred is the method in which the plants are
these genetically transformable agricultural and
horticultural species: corn, rice, orchardgrass,
soybean, cotton, Brassica (B. napus, B. campesteris,
25   B. olerica), potato, sugarbeet, pea (Pisum sativum)
alfalfa, sunflower, tobacco, flax, tomato, lettuce,
celery, carrot, eggplant, pepper (Capsicum annuum)
apple, melon, petunia, periwinkle, poplar and walnut.

Further, the male organ-specific promoter is the
30   TA29$_{1500}$ promoter, TA29$_{500}$ promoter (SEQ ID NO:1), or
promoters from Brassica anther-specific genes
represented by the cDNA clones p73, p112, p54, p42W,
p42, and p158 (SEQ ID NOS:2-7); the male organ is the
anther; the exogenous enzyme is β-glucuronidase (GUS);
35   and the protoxin is conjugated through a hydroxyl

residue to glucuronic acid. One skilled in the art,
given the disclosure herein, will be able to isolate the
promoters from the genes represent d by SEQ ID NOS:2-7
without undue experimentation.

5   This invention also pertains to protoxins that are
formed from herbicides, chemical hybridizing agents and
male sterilants linked to saccharides -- whose
conjugates are cleaved slowly or not at all in higher
plants -- via an oxygen atom. For simplicity hereafter,
10   the term "herbicide" (includes "herbicide derivative")
will be used to encompass herbicides, hybridizing agents
and sterilants. Preferred protoxins are herbicidal
sulfonylureas and maleic hydrazide linked to β-D-
glucuronic acid.

15   Contemplated protoxins are described hereafter. It
should be understood that glucuronic acid esters of
these protoxins are included within the scope of this
invention and that the term "protoxin" includes the
following compounds and their glucuronic acid esters.
20   In fact, an interesting aspect of this invention is the
use of the ester form of the protoxin, solely or in
various combinations with the free acid form, to induce
male sterility over a pre-targeted period of time during
which the male organ is known to develop. Extensive
25   plant esterase activity will release the free acid
glucuronide (or other non-ester form of the herbicide)
in a time-controlled manner to eliminate the need for
more than one application of protoxin even for plants in
which male fertility would otherwise normally develop
30   over several weeks.

           The protoxins of this invention are: (i)
derivatives of herbicidal sulfonylureas having a
glucuronic acid group added to any methyl, methylene or
methine carbon of the sulfonylurea, and (ii)
35   glucuronides of chemical hybridizing agents (CHA) such

as maleic hydrazide. The protoxin is a water-stable
compound in which the glucuronide is tether d with a 0
to 6, preferably 0 to 3, atom chain wh rein the chain is
made up of carbon atoms or, if the tether is 2 or more
5   atoms, one or more can be a sulfur, nitrogen or oxygen
atom.

A number of hydroxyl-containing sulfonylureas are
disclosed in the patents referred to in the "Background"
(supra). Additionally, hydroxyl-containing
10   sulfonylureas can be prepared by one skilled in the art
from the other sulfonylureas disclosed in said patents.

This invention employs sulfonylureas of Formula I,
including their agriculturally suitable isomers, salts
and derivatives thereof:

15

$$\underset{\underset{R}{|}}{\overset{\overset{W}{\|}}{JSO_2NHCNA}}$$

20

I

wherein J is

25

J-1   J-2   J-3

8

J-4   J-5

G is H or gluc-O(alkyl)$_n$L;

n is 0 or 1;

alkyl is 1 to 3 carbon atoms optionally substituted
     with one or two groups selected from halogen,
     methyl, methoxy or methylthio;

L is O, S(O)$_m$, NR^5, SO$_2$NR^4, CO$_2$, CH$_2$O, or a direct
     bond;

m is 0-3;

W is O or S;

R, R^4 and R^5 are independently H or CH$_3$;

E is a single bond or CH$_2$;

R^1 is H, C$_1$ to C$_3$ alkyl, C$_1$ to C$_3$ haloalkyl,
     halogen, nitro, C$_1$ to C$_3$ alkoxy, SO$_2$NR^{a}R^b,
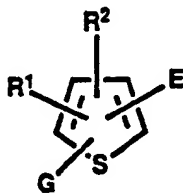   CONR^{a}R^b, C$_1$ to C$_3$ alkylthio, C$_1$ to C$_3$
     alkylsulfinyl, C$_1$ to C$_3$ alkylsulfonyl, CH$_2$CN, CN,
     CO$_2$R^c, C$_1$ to C$_3$ haloalkoxy, C$_1$ to C$_3$
     haloalkylthio, C$_2$ to C$_4$ alkoxyalkyl, C$_3$ to C$_4$
     alkoxyalkoxy, C$_2$ to C$_4$ alkylthioalkyl, CH$_2$N$_3$,
     NR^{d}R^e, or Q;

R^2 is H, C$_1$ to C$_3$ alkyl, C$_1$ to C$_3$ haloalkyl,
     halogen, nitro, C$_1$ to C$_3$ alkoxy, C$_1$ to C$_3$
     alkylthio, CN, C$_1$ to C$_3$ haloalkoxy, or C$_2$ to C$_4$
     alkoxyalkyl;

R^a is H, C$_1$ to C$_4$ alkyl, C$_2$ to C$_3$ cyanoalkyl,
     methoxy or ethoxy;

R^b is H, C$_1$ to C$_4$ alkyl or C$_3$ to C$_4$ alkenyl; or

R^a and R^b can be taken together as $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$ or $-CH_2CH_2OCH_2CH_2-$;

R^c is C_1 to C_4 alkyl, C_3 to C_4 alkenyl, C_3 to C_4 alkynyl, C_2 to C_4 haloalkyl, C_2 to C_3 cyanoalkyl, C_5 to C_6 cycloalkyl, C_4 to C_7 cycloalkylalkyl or C_2 to C_4 alkoxyalkyl;

R^d and R^e are independently H or C_1 to C_2 alkyl;

Q is a saturated or partially saturated 5- or 6-membered carbocyclic ring, containing either one or two carbonyl groups, or a saturated or unsaturated 5- or 6-membered heterocyclic ring, containing 1 to 5 atoms of carbon and 1 to 4 heteroatoms selected from the group consisting of 0 to 2 oxygen, 0 to 2 sulfur and 0 to 4 nitrogen, wherein sulfur can take the form of S, SO or SO_2, and containing 0 to 2 carbonyl groups; Q can further be optionally substituted with 1 to 2 substituent groups; substituents on carbon can be selected from the group consisting of halogen, C_1 to C_4 alkyl, C_1 to C_4 haloalkyl, $CH_2(C_2$ to C_3 alkenyl), $CH_2(C_2$ to C_3 alkynyl), C_2 to C_4 alkoxycarbonyl, CN, OH, C_1 to C_3 alkoxy, C_1 to C_3 alkylthio, C_1 to C_3 alkylsulfinyl, C_1 to C_3 alkylsulfonyl or C_2 to C_4 alkylcarbonyl; substituents on nitrogen can be selected from the group consisting of C_1 to C_4 alkyl, C_1 to C_4 haloalkyl, $CH_2(C_2$ to C_3 alkenyl), $CH_2(C_2$ to C_3 alkynyl), C_2 to C_4 alkoxycarbonyl or C_2 to C_4 alkylcarbonyl;

A is

X is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4 haloalkoxy, C_1 to C_4 haloalkyl, halogen, C_2 to C_5 alkoxyalkyl, C_2 to C_5 alkoxyalkoxy, amino, C_1 to C_3 alkylamino, di(C_1 to C_3 alkyl)amino, C_3 to C_5 cycloalkyl, C_1 to C_4 alkyl substituted with —O-gluc, C_2 to C_4 alkoxyalkyl substituted with —O-gluc, or C_1 to C_4 alkoxy substituted with —O-gluc;

Y is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4 haloalkoxy, C_2 to C_5 alkoxyalkyl, C_2 to C_5 alkoxyalkoxy, amino, C_1 to C_3 alkylamino or di(C_1 to C_3 alkyl)amino;

R^3 is H or C_1 to C_3 alkyl;

Z is CH or N;

E^1 is a direct bond or CH_2;

gluc is β-D-glucuronic acid;

provided that:

    (i)   when G is H then X is C_1 to C_4 alkyl substituted with gluc, or C_1 to C_4 alkoxy substituted with gluc;

    (ii)   when L is not a direct bond, then n is not zero and alkyl comprises at least two carbon atoms which can be substituted as described.

Preferred are compounds of Formula I wherein

W is O;

R^1 is H, halogen, C_1 to C_3 haloalkoxy, $SO_2NR^aR^b$ $CONR^aR^b$, CO_2R^c, C_3-C_4 alkoxyalkoxy or Q;

R^2 is H, methyl, halogen, methoxy or
trifluoromethyl;

R^a and R^b are, independently, H or C_1 to C_4 alkyl,
or R^a and R^b can be taken together as $-(CH_2)_4-$,

5          $-(CH_2)_5-$ or $-CH_2CH_2OCH_2CH_2-$;

R^c is C_1 to C_4 alkyl, C_2 to C_4 haloalkyl or C_2 to C_4
alkoxyalkyl;

X is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4
haloalkyl, C_1 to C_4 haloalkoxy, halogen, C_2 to C_5

10   alkoxyalkyl, C_2 to C_5 alkoxyalkoxy, C_1 to C_3
alkylamino, C_1 to C_4 alkyl substituted with
$-O-gluc$, C_2 to C_4 alkoxyalkyl substituted with
$-O-gluc$ or C_1 to C_4 alkoxy substituted with
$-O-gluc$.

15 More preferred are compounds of Formula I wherein:

R^1 is H, halogen, $SO_2NR^aR^b$, $CONR^aR^b$, CO_2R^c or
tetrazolyl;

J is J-1, J-2, J-3 or J-4;

G is H, gluc-O- or gluc-O-(alkyl)L;

20   L is O, $S(O)_m$, CO_2, NR^5 or a direct bond; and m is
0 or 2.

Still more preferred are compounds of Formula I wherein:

R^1 is H, Cl, $CONR^aR^b$ or CO_2R^c;

J is J-1 or J-4;

25   G is H, gluc-O- or gluc-O-CH_2CH_2-L

L is O, S, SO_2, NR^5 or CO_2;

E is a single bond;

R^a and R^b are, independently, H or C_1 to C_4 alkyl;

R^c is C_1 to C_4 alkyl;

30   X is H, C_1 to C_3 alkyl, C_1 to C_3 alkoxy, Cl, C_1 to
C_3 alkylamino or C_1 to C_3 alkyl substituted with
$-O-gluc$, C_2 to C_3 alkoxyalkyl substituted with
$-O-gluc$; or C_1 to C_3 alkoxy substituted with
$-O-gluc$; and

Y is C_1 to C_3 alkyl, C_1 to C_3 alkoxy, C_1 to C_3 haloalkoxy, C_1 to C_3 alkylamino or C_1 to C_3 alkyl.

Most preferred are the glucuronides of

N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-6-[(2-hydroxyethyl)methylamino]-2-pyridine-sulfonamide; and

2-[(2-hydroxyethyl)thio]N-[[(4-methoxy-6-methyl-2-pyrimidinyl)amino]-carbonyl]benzenesulfonamide.

This invention also pertains to the recited plants containing a novel DNA construct comprising a male organ-specific promoter operably linked upstream from GUS. Preferred are transgenic plants containing the $TA29_{1500}$ or $TA29_{500}$ promoter linked to GUS. This invention also pertains to the seeds produced by the transgenic plants and all progeny that exhibit the desirable traits herein described. This invention also pertains to vectors containing a novel DNA construct comprising a particular male organ-specific promoter operably linked upstream from GUS. Preferred is the PZS96 *Agrobacterium* binary vector containing the NptII marker gene and the $TA29_{1500}$ or $TA29_{500}$ promoter linked to GUS. This invention also pertains to a method of hybrid seed production and to the transgenic plants that have been exposed to the glucuronide conjugates. Finally, this invention concerns the promoter $TA29_{500}$ characterized by its ability to reliably direct anther specific expression in transgenic plants. $TA29_{500}$ comprises a nucleic acid fragment derived from the TA29 gene that extends from the EcoRV restriction site, which is about 500 base pairs 5' to the transcription start site and extending to the translation initiation ATG.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the maps of _Agrobacterium tumefaciens_ binary vectors containing the TA29/P-GUS-Nos 3' chimeric genes. A) pZ6ASG: The 500 bp EcoRV-NcoI
5 TA29 promoter combined with the GUS coding region and the Nos 3' region as described in Example 1 is adjacent to the NosP-NptII-Ocs 3' selection marker gene. Both of these genes are between the T-DNA borders in the binary vector pZS96, which includes a gene for ampicillin
10 resistance (Amp^R) and an origin of replication from pBR322 (ori), and the replication (rep pVS1) and stability (sta) regions from pVS1. B) pZ6ALG: As in A) except that the 1500 bp ClaI-NcoI TA29 promoter is combined with GUS-Nos 3' as described in Example 2.

15   Figure 2 shows the GUS enzyme activity assayed in anthers of transgenic plants containing the TA29$_{500}$/P-GUS-Nos 3' gene as described in Example 3. Figure 2A represents a wild type tobacco plant, which shows virtually no endogenous GUS activity. Figures 2B
20 and 2C show the anther-specific enzyme activity in two independent transformants that demonstrate anther-specific expression of GUS. Substantially no activity was observed in wild-type or transformed plant ovules or leaves (old or new).

25   DETAILS OF THE INVENTION

Definitions

Terms that are used frequently throughout the description are defined as follows:

"Male sterility" of a plant refers to the inability
30 of the plant to produce fertile, functional pollen to germinate on the stigma and to effect the fertilization of the egg.

"Male organ" of the plant refers to the part of the flower that physically contains pollen. Pollen grains,
35 at all stages of development, are considered a part of

the male organ. In general, male organ denotes "stamen" which includes the filament, anther and pollen in the anther. For the sake of simplicity, the term is intended to include the "gamete" as well. By "gamete"

5 is meant a mature germ cell capable of forming a new individual by fusion with another gamete.

"Tapetum" refers to the innermost layer of anther wall which surrounds the developing pollen. At a late stage of pollen maturation, the tapetum layer

10 disintegrates entirely.

"DNA-construct" refers to a linear or circular molecule of deoxyribonucleic acid (DNA), that is a composition of DNA fragments derived from any source.

An "exogenous enzyme" refers to an enzyme produced

15 according to the information in the coding region of a gene which is introduced by transformation into the plant.

A "chemical hybridizing agent" (CHA) means a chemical that can render a plant partially or fully male

20 sterile when it is applied to the plant.

The term "protoxin" refers to a chemical that releases a toxin upon reaction with the exogenous enzyme. Without interacting with the exogenous enzyme, the protoxin can also be converted into the toxin under

25 certain chemical conditions. An agent that is "toxic" to the male organ refers to an agent that kills or renders the male organ non-functional. It may cause the production of dead pollen, or living but non-functional pollen, or both. The living but non-functional pollen

30 cannot effect egg fertilization when applied to the stigma of the pistil.

In the molecular sense, the term "hybridization" means the bonding of complementary segments of DNA to DNA or RNA to DNA.

The term "hybridization", in the biological sense, refers to the production of offspring by crossbreeding of two plants that ar genetically different.

The "female parent" in hybridization means the
5   plant whose eggs in the ovary of the flower are fertilized by the pollen of the male parent.

"Beta-glucuronidase" (GUS) is any enzyme that catalyzes the hydrolysis of β-O-glucuronide conjugates into glucuronic acid and the aglycone. In particular,
10 GUS catalyzes the hydrolysis of a glucuronide conjugate protoxin into the toxin and glucuronic acid.

The term "glucuronide" is used interchangeably with "beta-glucuronide". It denotes a derivative of glucuronic acid in which a compound (the aglycone) is
15 conjugated through the beta linkage to the oxygen atom on carbon number 1 of the glucuronic acid.

The "aglycone" of the glucuronide refers to any compound that is conjugated to the glucuronic acid. The chemical nature of the aglycone is limited only by the
20 requirement that it possess a conjugatable hydroxyl group.

The expression "nucleotide sequence" refers to a polymer of DNA or RNA, which can be single- or double-stranded, optionally containing synthetic, non-natural,
25 or altered nucleotides capable of incorporation into DNA or RNA polymers.

"DNA segment" refers to a linear fragment of single- or double-stranded DNA derived from any source. The expression "DNA in plant cells" includes all DNA
30 present in plant cells. As used herein, a "gene" is intended to mean a DNA segment which includes a 5' regulatory region, a coding region, and a 3' polyadenylation nucleotide region and is normally regarded as a gene by those skilled in the art.

"Coding region" refers to a DNA segment which encodes a regulatory molecule or any polypeptide.

"Gene product" refers to a polypeptide resulting from transcription, translation, and, optionally, post-

5 translational processing of a selected DNA segment.

The term "expression" as used herein is intended to mean the translation to gene product from a gene coding for the sequence of the gene product. In expression, a DNA chain coding for the sequence of gene product is

10 first transcribed to a complementary RNA which is called a messenger RNA and then, the thus transcribed messenger RNA is translated into the above-mentioned gene product.

The term "promoter" or "promoter region" refers to a sequence of DNA, usually upstream (5') of the coding

15 sequence, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at the correct site. A "promoter fragment" constitutes a DNA sequence consisting of the promoter region. A

20 promoter region can include one or more regions which control the effectiveness of transcription initiation in response to physiological conditions, and a transcription initiation sequence.

"Tissue specific promoters" are those that direct

25 gene expression primarily in specific tissues.

A "male organ-specific promoter" refers to a promoter that directs gene expression primarily in the male organ, i.e., pollen, anther tissues, filament of the anther, and gamete. Transcription stimulators,

30 enhancers or activators can be integrated into these promoters to create a promoter with a high level of activity that retains its specificity.

"Regulatory nucleotide sequence" refers to a nucleotide sequence located proximate to a coding region

35 whose transcription is controlled by the regulatory

nucleotide sequence in conjunction with the gene
xpression apparatus of th cell. Generally, th
regulatory nucleotide s quence is located 5' to the
coding region. A promoter can include one or more
5 regulatory nucleotide sequences.

"Polyadenylation nucleotide sequence" (or "region")
refers to a nucleotide sequence located 3' to a coding
region which controls the addition of polyadenylic acid
to the RNA transcribed from the coding region in
10 conjunction with the gene expression apparatus of the
cell.

"Transformation" means the process by which
cells/tissues/plants acquire properties encoded on a
nucleic acid molecule that has been transferred to the
15 cell/tissue/plant. "Transferring" refers to methods to
transfer DNA into cells including, but not limited to,
microinjection, permeabilizing the cell membrane with
various physical (e.g., electroporation) or chemical
(e.g., polyethylene glycol, PEG) treatments, high-
20 velocity microprojectile bombardment also termed
biolistics, or infection with _Agrobacterium_ _tumefaciens_
or _A._ _rhizogenes_.

"Transformant" or "transgenic plant" means a plant
which has acquired properties encoded on a nucleic acid
25 molecule that has been transferred to cells during the
process known as transformation.

"Integrated" means that the DNA is incorporated
into the plant genome.

In the present invention, the β-glucuronidase
30 enzyme is specifically produced in anther or pollen
cells. GUS catalyzes the hydrolysis of a wide variety
of glucuronides. Most any aglycone conjugated to D-
glucuronic acid through a β-O-glycosidic linkage is a
suitable substrate. GUS enzyme activity can be easily
35 assayed using a number of methods including

spectrophotometrically using p-nitrophenyl-glucuronide as the substrate, fluorometrically using 4-methyl umbelliferyl-glucuronide (MUG) as th substrate, or histochemically using 5-bromo-4-chloro-3-indolyl-

5 glucuronide (X-Gluc) as the substrate.

The longevity, broad pH optimum, broad substrate range, good heat tolerance, lack of co-factors, and particularly the absence of measurable amounts of the enzyme in higher plants combine to make β-glucuronidase

10 a very desirable enzyme to generate a toxin from a glucuronide protoxin.

The GUS enzyme is naturally encoded by the uidA gene of Escherichia coli. The cloning and characterization of this gene, and subcloning of the

15 coding region, is described by Jefferson et al., Proc. Natl. Acad. Sci. USA 83: 8447-8451 (1986). The use of this coding region in conjunction with various promoter regions, thereby providing the GUS enzyme as a marker for their expression, is well established in the art,

20 for example, in Jefferson et al., EMBO J 6: 3901-3907 (1987), Benfey et al., EMBO J 8: 2195-2202 (1989), Broglie et al., The Plant Cell 1: 599-607 (1989), Keil et al., EMBO J 8: 1323- 1330 (1989), and Keller et al., EMBO J 8: 1309-1314 (1989). A chimeric gene composed of

25 the GUS coding region and the CaMV 35S promoter is available from Clontech, Inc.

The GUS enzyme is expressed as the product of a chimeric gene that is transferred into plants, that includes a regulatory nucleotide sequence which directs

30 expression specifically in cells located in the anther or in pollen. Suitable regulatory nucleotide sequences are known in the art. The particular anther or pollen-specific promoter which is employed with a selected plant species is not critical to the method of the

35 invention. A partial list of suitable promoters

includes those from the TA29 and TA13 tobacco genes described by Goldberg, _Science_, 240: 1460-1467 (1988), the LAT52 tomato g ne d scribed by Twell et al, _Mol. Gen. Genet._ 217: 240-245 (1989), the Zmc13 and pZmc26

5   corn genes described by Stinson et al., _Plant Physiol._ 83: 442-447 (1987), the pTpc70 and pTpc44 _Tradescantia_ genes also described by Stinson et al., and the CHI-A (P_{A2} region) and CHI-B petunia genes described by van Tunen et al., _Plant Mol. Biol._ 12: 539-551 (1989).

10 Novel anther promoters are derived from _Brassica napus_ genes corresponding to the newly-identified anther-specific cDNAs with SEQ ID NOS:2-7. Most preferably, the regulatory nucleotide sequence is a TA29 promoter.

Methods for introducing a DNA sequence into plant

15 cells are known in the art. Nucleic acids can generally be introduced into plant protoplasts, with or without the aid of electroporation, polyethylene glycol, or other processes known to alter membrane permeability. Nucleic acid constructs can also be introduced into

20 plants using vectors comprising part of the Ti- or Ri-plasmid, a plant virus, or an autonomously replicating sequence. Nucleic acid constructs can also be introduced into plants by microinjection or by high-velocity microprojectiles, also termed "particle

25 bombardment" or "biolistics" (Sanford, _Tibtech_ 6: 299 (1988)), directly into various plant parts. The preferred means of introducing a nucleic acid fragment into plant cells involves the use of _Agrobacterium tumefaciens_ containing the nucleic acid fragment between

30 T-DNA borders in a binary vector _in trans_ to a disarmed Ti-plasmid. The _Agrobacterium_ can be used to transform plants by inoculation of tissue explants, such as stems, roots, or leaf discs, or by co-cultivation with plant protoplasts.

The range of crop species in which foreign genes
can b introduced is increasing rapidly as tissue
culture and transformation methods improve and as
appropriate selectable markers become available. Thus,
5   this invention is applicable to a broad range of
agronomically or horticulturally useful plants. The
particular method which is employed to introduce the DNA
sequence into a selected plant cell is not critical. In
a preferred embodiment, DNA sequences are introduced
10   into plant cells by co-cultivation of leaf disks or
plant tissue explants with _Agrobacterium tumefaciens_.

Most preferably, the plasmid for introducing a DNA
sequence comprising an anther or pollen specific
promoter, a GUS coding region, and a nopaline synthase
15   gene polyadenylation nucleotide sequence (NOS 3') is
pZ6ASG or pZ6ALG or derivatives thereof. These plasmids
can be used to generate plants that express the GUS
enzyme in their anthers by those skilled in the art or
as taught in this application.

20   In the present method, plants expressing GUS in the
anthers or pollen are contacted with a protoxin, such as
a general cytotoxic agent or a herbicide that has been
conjugated through an oxygen atom to glucuronic acid.
The protoxin is transported to all regions of the plant,
25   but is efficiently cleaved to toxin only in the male
organs and exhibits cytotoxicity only to the male
organs. The plant is thus rendered male-sterile upon
exposure to the protoxin. As will be appreciated by one
skilled in the art, not all protoxins will be equally
30   effective as male-sterilants in all plants that have
anther-specific expression of GUS. Some of them may be
toxic to certain plants. Others may have relatively low
toxicity to the male organ. Nevertheless, given the
disclosure presented herein, and with a minimum of
35   experimentation, one skilled in the art will be able to

easily determine which protoxin(s) to employ with a
sp cific plant.

EXAMPLES

Methods of culturing bacteria, preparing DNA, and
5   manipulating DNA were as described by Maniatis et al.,
Molecular Cloning: A Laboratory Manual [Cold Spring
Harbor Laboratory, New York (1982)] unless stated
otherwise. Restriction enzymes and other enzymes used
in DNA manipulations were obtained from New England
10   Biolabs, Inc. (Beverly, MA, USA), Boehringer Mannheim
(Indianapolis, IN, USA), or Bethesda Research Laboratory
(Gaithersburg, MD, USA) and were used according to the
manufacturer's specifications.

EXAMPLE 1

15   A chimeric gene was constructed to obtain
expression of the β-glucuronidase (GUS) coding region
in the tapetal cells of anthers. The promoter was
derived from the tobacco TA29 gene, a gene that is
expressed naturally only in the tapetal tissue of the
20   tobacco anther. A clone containing the TA29 gene was
obtained from Dr. Goldberg at the University of
California, Los Angeles. The TA29 gene can also be
obtained by methods taught in EPA 84-344,029. One
skilled in the art can prepare a probe to the TA29 cDNA
25   sequence given in Figure 2 of '029 and isolate a TA29
gene-containing clone from a tobacco genomic library
using that probe. The TA29 gene sequence is given in
Fig. 3 of EPA 89-344029.

A TA29 promoter fragment was prepared from the
30   TA29 gene by first cloning an SstI-HindIII fragment,
which was expected to contain the TA29 promoter region
based on the location of the 5' end of the messenger
RNA, from the Goldberg clone into the SstI and HindIII
digested vector M13mp18. This fragment proved too
35   large to carry out further steps so an approximately

500 base pair (bp) EcoRV-HindIII fragment was isolated and cloned into SmaI and HindIII digested M13mp19. (EcoRV and SmaI both leave blunt nds.) The translation initiation ATG was identified and the

5   sequence of DNA surrounding it was determined by sequencing in from the HindIII end of the fragment according to the method of Sanger et al., Proc. Natl. Acad. Sci. USA 74: 5463-5467 (1977) using a U.S. Biochemical Corporation Sequenase DNA sequencing kit

10   and following the manufacturer's protocol. Next, an oligonucleotide with the sequence AGAAATTAGCTACCATGGTAGCTCCAAAAT (SEQ ID NO:8) was synthesized using an Applied Biosystems DNA synthesizer and following the manufacturer's procedure. This

15   oligonucleotide was used in a site-directed mutagenesis procedure as described in Viitanen et al., J. Biol. Chem., 263:15000-15007 (1988), to create an NcoI site surrounding the translation initiation ATG. The approximately 500 bp TA29 promoter fragment containing

20   the new NcoI site was then moved as an SstI-HindIII fragment, the SstI site being derived from the M13mp19 polylinker, into SstI and HindIII digested pTZ19 (available from Pharmacia) creating pTZAS. Next, an NcoI-HindIII fragment that includes the GUS coding

25   region and a nopaline synthase gene polyadenylation nucleotide sequence (NOS 3') was prepared from the plasmid pTZCGNC.

The pTZCGNC vector contains pTZ19 and a chimeric NOS/P-GUS-NOS 3' gene and was constructed in the

30   following manner. A GUS coding region fragment was prepared from pRAJ275, which is described in Jefferson et al., Proc. Natl. Acad. Sci. USA 83, 8447-8451 (1986) and is available from Clontech Laboratories. The plasmid, pRAJ275, was digested with EcoRI, the end made

35   blunt, and a BamHI linker was added. It was then

digested with HindIII to prepare a HindIII-BamHI
fragment containing the GUS coding region. This
fragment was ligated into HindIII and BamHI digested
pKNK creating pKNGUS. The plasmid, pKNK, bears ATCC

5   deposit accession number 67284. It is a pBR322 based
vector which contains a neomycin phosphotransferase II
(NptII) promoter fragment, a nopaline synthase (NOS)
promoter fragment, the coding region of NptII and the
polyadenylation nucleotide sequence from the NOS gene.

10 A map of this plasmid is shown in Lin et al., _Plant_
Physiol. 84: 856-861 (1987). The 320 bp ClaI-BglII
fragment in pKNK that contains the NptII promoter was
obtained as a HindIII-BglII fragment from the NptII
gene of the transposon Tn5 described by Beck et al.,

15 _Gene_ 19: 327-336 (1982). The HindIII site was
converted to a ClaI site by linker addition. The NptII
promoter fragment is followed by a 296 bp Sau3A-PstI
NOS promoter (NOS/P) fragment corresponding to
nucleotides -263 to +33, with respect to the

20 transcription start site, of the NOS gene described by
Depicker et al., _J._ _Appl._ _Genet._ 1: 561-574 (1982).
The PstI site at the 3' end was created at the
translation initiation codon of the NOS gene. The
NOS/P is followed by a 998 bp HindIII-BamHI sequence

25 containing the NptII coding region obtained from the
transposon Tn5 [Beck et al., _Gene 19_: 327-336 (1982)]
by the creation of HindIII and BamHI sites at
nucleotides 1540 and 2518, respectively. The NptII
coding region is then followed by a 702 bp BamHI-ClaI

30 fragment containing the 3' end of the nopaline synthase
gene including nucleotides 848 to 1550 [Depicker et
al., _J._ _Appl._ _Genet._ 1: 561-574 (1982)]. The remainder
of pKNK consists of pBR322 sequences from 29 to 4361.
      From pKNGUS, a ClaI fragment containing the

35 chimeric NOS/P-GUS-NOS 3' gene was isolated, the ends

were made blunt, and it was ligated into the SmaI site
of pTZ19 creating pTZCGNC. An NcoI-HindIII fragment
containing the GUS coding r gion and NOS 3' was
isolated, the HindIII site being derived from the pTZ
5 polylinker, and it was ligated into NcoI and HindIII
digested pTZAS creating the chimeric $TA29_{500}$/P-GUS-NOS
3' gene in the plasmid pTZASG. The resulting plasmid
contains a chimeric gene that has a 500 bp TA29
promoter and a NOS 3' as the regulatory signals
10 surrounding the GUS coding region in the vector pTZ19.
        This chimeric gene was transferred into pZS96, a
binary vector used in Agrobacterium tumefaciens
transformations of plant tissue. The plasmid, pZS96,
contains the origin of replication and ampicillin
15 resistance gene from pBR322 for maintenance and
selection in E. coli. It contains the replication and
stability regions of the Pseudomonas aeruginosa plasmid
pVS1, described by Itoh et al., Plasmid 11: 206-220
(1984), which are required for replication and
20 maintenance of the plasmid in Agrobacterium. Also
contained are a T-DNA left border fragment of the
octopine Ti plasmid pTiA6 and a right border fragment
derived from TiAch5 described by van den Elzen et al.,
Plant Molec. Biol. 5: 149-154 (1985). Between these
25 borders are a NOS/P-NptII-OCS 3' chimeric gene to
confer kanamycin resistance to plant cells and a LacZ
gene with the unique restriction sites HindIII, SalI,
BamHI, SmaI, KpnI, and EcoRI derived from pUC18. pZS96
was digested with SmaI and HindIII and ligated to a
30 ScaI-HindIII fragment containing the $TA29_{500}$/P-GUS-NOS
3' gene isolated from pTZAS, the ScaI site being
located in the pTZ sequence. (ScaI and SmaI digests
both leave blunt ends.) The resulting plasmid called
pZ6ASG (Figure 1A) contains a chimeric gene that has a
35 549 bp TA29 promoter (SEQ ID NO:1) and a NOS 3' as the

regulatory signals surrounding the GUS coding region in
the vector pZS96.

<div align="center">

EXAMPLE 2

</div>

A second promoter fragment from the TA29 gene was
5   prepared by first isolating an approximately 1500 bp
ClaI-HindIII fragment from the SstI-HindIII fragment
that was subcloned from the Goldberg TA29 gene clone
described above. During the isolation, the ClaI end
was filled in so that this fragment could be cloned
10 into the HincII (blunt end) and HindIII sites of
M13mp19. The same method described above was used to
create an NcoI site surrounding the translation
initiation ATG. The TA29 promoter fragment containing
the new NcoI site was then moved as a SmaI-HindIII
15 fragment, the SmaI site being derived from the M13mp19
polylinker, into SmaI and HindIII digested pTZ19
creating pTZAL. Before adding the GUS-NOS 3' fragment
adjacent to the promoter, the ClaI fragment containing
the NOS/P-GUS-NOS 3' gene in pTZCGNC was isolated, the
20 ends made blunt, and it was cloned into SphI digested
and blunted pTZ19. This step was carried out to
eliminate the polylinker sites that are located between
the NOS 3' and the HindIII site in pTZASG. The NcoI-
HindIII fragment that includes the GUS-NOS 3' was
25 prepared from the resulting plasmid and it was cloned
into NcoI and HindIII digested pTZAL creating pTZALG.
An Asp718-HindIII fragment containing the entire
chimeric TA29$_{1500}$/P-GUS-NOS 3' gene was ligated into
Asp718 and HindIII digested pZS96, described above,
30 creating pZ6ALG (Figure 1B). The resulting plasmid is
unique in that it contains a chimeric gene that has
with the 1500 bp TA29 promoter and GUS coding region, a
NOS 3' as the polyadenylation regulatory signal, and
this chimeric gene is present in the pZS96 vector.

EXAMPLE 3

The chimeric TA29$_{500}$/P-GUS-NOS 3' gene, described
above, was introduced into tobacco by Agrobacterium
tumefaciens infecti n of leaf disks. Primary
5   transformants were analyzed to demonstrate anther
specific expression of this gene.

The plasmid pZ6ASG was transferred into
Agrobacterium tumefaciens by a method involving a
three-way mating that was essentially as described by
10   Fraley et al., Proc. Natl. Acad. Sci. USA, 80: 4803-
4807 (1983) except for the following points. The
plasmid, pZ6ASG, was mated into Agrobacterium strain
LBA4404 described by Hoekema et al., Nature 303: 179-
180 (1983). Colonies from the pZ6ASG mating were
15   selected on MinA (Table 1) plates containing 100 μg/mL
rifampicin, 25 μg/mL carbanicillin, and 10 μg/mL
tetracycline. Selected colonies were confirmed as
transformants by restriction digests of miniprep DNA.
To prepare a culture for plant transformation, 5 mL of
20   MinA liquid medium (without agar) was inoculated with a
single Agrobacterium colony harboring pZ6ASG and grown
for approximately 17-20 hours in 18 mm glass culture
tubes in a New Brunswick platform shaker maintained at
28°C.

25   Standard aseptic techniques for the manipulation
of sterile media and axenic plant/bacterial cultures
were followed, including the use of a laminar flow hood
for all transfers. Potted tobacco plants for leaf disk
infections were grown in a growth chamber maintained
30   for a 14 hr, 21°C day, 10 hr, 18°C night cycle, with
approximately 80% relative humidity, under mixed cool
white fluorescent and incandescent lights. Tobacco
leaf disk infection was carried out essentially by the
method of Horsch et al., Science 227: 1229-1231 (1985),
35   omitting nurse cultures, as described below.

Healthy young leaves, not fully expanded and
approximat ly 4-5 inches in length, were harv sted from
approximately 5-7 week old tobacco plants (<u>Nicotiana
tabacum</u> var. Xanthi). The leaves were surface

5   sterilized for 30 minutes by immersion in a solution
containing 10% Clorox® and 0.1% sodium dodecyl sulfate
(SDS) and then rinsed three times with sterile
deionized water. Leaf disks, 8 mm in diameter, were
prepared from whole leaves using a sterile paper punch.

10   Leaf disks were inoculated by submerging them for
several minutes in 20-30 mL of a 1:10 dilution of the
overnight culture of <u>Agrobacterium</u> harboring the pZ9ASG
plasmid. After inoculation, the leaf disks were placed
on CN agar medium (Table 1) in petri dishes which were

15   then sealed with parafilm. The petri dishes were
incubated under mixed fluorescent and grow lights for
2-3 days in a culture room maintained at approximately
25°C.

     To rid the leaf disks of <u>Agrobacterium</u> and to

20   select for the growth of transformed tobacco cells, the
leaf disks were transferred to fresh CN agar medium
containing 500 mg/L cefotaxime and 100 mg/L kanamycin.
Leaf disks were incubated under the growth conditions
described above for 3 weeks and then transferred to

25   fresh media of the same composition.

     Approximately 2 weeks later, shoots developing on
kanamycin-containing medium were excised with a sterile
scalpel and planted in Root Induction medium 'A' (Table
1) containing 500 mg/L cefotaxime and 100 mg/L

30   kanamycin. Root formation was recorded within 3 weeks.
Rooted shoots were then transplanted to wet Metro-Mix
in 8-inch pots, moved to a growth chamber set at the
conditions described above, and covered with plastic
bags for 7-10 days. After 4-6 weeks, plants were

transferred to the greenhouse and allowed to grow to
maturity.

To assess th anther-specific expression of the
introduced chimeric TA29$_{500}$/P-GUS-NOS 3' gene different
5 plant tissues were tested for GUS enzyme activity as
follows. For each plant, protein extracts were made
from the collected five anthers of a young flower bud
of stage 3-4 [the time of peak TA29 expression
according to Goldberg, Science 240: 1460-1467 (1988)],
10 from the ovule and pistil, from 0.05 g of tissue from a
young leaf, and from 0.05 g of tissue from an old leaf.
Each tissue sample was ground on ice in a microfuge
tube containing 125-200 μL of GUS extraction buffer (50
mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% triton
15 X-100, 0.1% sarkosyl, 10 mM β-mercaptoethanol) with a
Kimble pellet pestle. The extracts were centrifuged in
a microfuge for 5 minutes, and the supernatants
transferred to new tubes. Then, 150 μL of each
supernatant (with more GUS extraction buffer added if
20 necessary) was transferred to another tube, 16.7 μL of
a 10 mM 4-methyl umbelliferyl-glucuronide (MUG)
solution in GUS extraction buffer was added as the GUS
enzyme substrate, mixed, and 30 or 40 μL samples were
transferred to a microtitre plate as time point samples
25 at 0, 2, and 4 hours or at 10, 20, 30, and 60 minute
intervals. Each well in the microtitre plate was
previously filled with 360 μL of 0.2 M sodium carbonate
to stop the reaction. Fluorescence of the reaction
product, 4MU, was measured using a Perkin-Elmer plate
30 reader with the excitation setting at 365 nm and the
reading taken at 455 nm. The protein concentration of
each supernatant extract was determined using the
BioRad Protein Assay reagent according the the
manufacturer's protocol. The data was compiled as

nanomoles of 4MU produced per mg of protein at each
time point.

Sixteen independent tobacco transformants were
assayed as described above with the following results.
5   56% of the plants (9/16) showed tightly regulated
anther specific expression of the TA29$_{500}$/P-GUS-NOS 3'
gene in that the ovule, new leaf, and old leaf samples
had essentially no GUS activity, as seen in the wild
type control tobacco plant samples, and substantial GUS
10   activity was present in the anther sample (Figure 2).
Twenty-five percent of the plants (4/16) showed
substantial GUS activity in the anther sample, but the
level in one or more of the other tissues tested was
greater that that in the control. This category of
15   transformant was classified as having anther specific
expression with high background. One plant (6%) had
non-specific activity in that the anther GUS activity
was lower than in other samples. Finally, 13% of the
plants (2/16) had no GUS activity. These results
20   indicate that though not every transformant exhibits
the desired pattern of TA29$_{500}$/P-GUS-NOS 3' expression,
the 500 bp TA29 promoter fragment is capable of
directing anther specific expression of GUS in
individual transformed tobacco plants.
25   To verify that anthers of buds at stages 3 and 4
show the peak TA29$_{500}$/P-GUS-NOS 3' expression levels, a
time course of GUS activity was established in anthers
from two plants exhibiting anther specific GUS
expression. Anthers from buds of stages 2,3,4, and 6
30   were assayed for GUS enzyme activity using the
fluorescent assay described above. In each case the
activity was highest at stage 3.

Seeds were collected following self fertilization
of individual transformants. To determine the number
35   of genetic loci for the TA29$_{500}$/P-GUS-NOS 3' gene, and

linked kanamycin resistance marker, s eds were
germinated on plat s of Root Induction medium 'A'
containing 100 or 200 µg/mL kanamycin. S eds wer
sterilized by incubation for 30 minutes in 10% Clorox®

5   and 0.1% SDS and sewn at 60 seeds per plate. Sensitive
seeds germinate, but the seedlings bleach after 2-3
weeks. A segregation ratio of 3 resistant to 1
sensitive indicates the presence of a single site of
integration of the kanamycin resistance gene into the

10   genome of the transformant, which is then stably
inherited by its progeny. This was seen in 3 out of 6
of the transformants with anther specific GUS
expression that were tested. The remaining
transformants exhibited a ratio which was greater than

15   3:1, indicating the presence of more than one
integration site.

        To establish lines of plants homozygous for the
TA29$_{500}$/P-GUS-NOS 3' gene, 4-7 kanamycin resistant
plants from lines showing 3:1 segregation in the seed

20   germination test were potted in soil and grown to
maturity in the greenhouse. Seed were collected after
self fertilization and germinated on kanamycin medium
as described above. Populations of seed that were 100%
kanamycin resistant representing homozygous lines were

25   identified.

TABLE 1

MinA

Per 500 mL: 100 mL: 5.25 g K_2HPO_4, 2.25 g KH_2PO_4,

30   0.5 g $(NH_4)_2 \cdot SO_4$, 0.25 g sodium
                           citrate.$2H_2O$
                   0.5 mL of 0.2 g/mL $MgSO_4 \cdot 7H_2O$
                   5 mL of 20% sucrose or glucose
                   7.5 g agar

Leaf Shoot Medium 'CN'
Per liter: 1 pkg MS Minimal Organic Medium with
                          sucrose   (Gibco)
                  ·1 mL of 1 mg/mL benzylaminopurine (BAP)
5   pH 5.6
                  8 g agar
Root Induction Medium 'A'
Per liter: 1 pkg MS Minimal Organic Medium without
                          sucrose (Gibco)
10   10 g sucrose
                  pH 5.6
                  8 g agar
All of the above can be autoclaved after adding
hormones.

15   EXAMPLE 4

The chimeric $TA29_{1500}$/P-GUS-NOS 3' gene was
introduced into tobacco by _Agrobacterium_ _tumefaciens_
infection of leaf disks as described above using
Agrobacterium harboring the pZ6ALG plasmid. Primary

20 transformants were analyzed to demonstrate anther
specific expression of the introduced chimeric GUS gene
using the enzyme fluorescence assay as described above.

The twenty-four independent tobacco transformants
that were assayed were placed in the same categories

25 described for the $TA29_{500}$/P-GUS-NOS 3' transformants.
Forty-two percent of the plants (10/24) showed tightly
regulated anther specific expression of the $TA29_{1500}$/P-
GUS-NOS 3' gene; 17% of the plants (4/24) had anther
specific expression with high background; 25% of the

30 plants (6/24) had non-specific activity. Finally, 17%
of the plants (4/24) had no GUS activity. These
results indicate that though not every transformant
exhibits the desired pattern of $TA29_{1500}$/P-GUS-NOS 3'
expression, the 1500 bp TA29 promoter fragment is

35 capable of directing anther specific expression of GUS

in individual transformed tobacco plants. Two of the
plants with anther specific GUS expression showed much
higher lev ls of GUS activity in the anthers than seen
for any of the TA29$_{500}$/P-GUS-NOS 3' transformants.

5   The anther specific GUS expression was further
characterized as being limited to tapetal cells by
incubating anthers in 0.1 mM NaPO$_4$ buffer, pH 7.0, 0.5
mM K$_3$[Fe(CN)$_6$], 0.5 mM K$_4$[Fe(CN)$_6$]·3H$_2$O, 10 mM Na$_2$EDTA
containing 1 mg/mL X-Gluc (5-bromo-4-chloro-3-indolyl-
10 β-glucuronide). This GUS substrate releases a blue
product upon GUS hydrolysis. Microscopic observation
showed that the blue staining was limited to the
tapetal cells in the anther. Wild-type control tobacco
anthers showed no blue staining.

15   To verify that anthers of buds at stages 3 and 4
show the peak TA29$_{500}$/P-GUS-NOS 3' expression levels, a
time course of GUS activity was established in anthers
from two plants exhibiting anther specific GUS
expression. Anthers from buds of stages 2,3,4, and 5
20 were assayed for GUS enzyme activity using the
fluorescence assay described above. In each case the
activity was highest at stage 3.

     Seeds were collected following self-fertilization
of individual transformants. To determine the number
25 of genetic loci for the TA29$_{1500}$/P-GUS-NOS 3' gene, and
linked kanamycin resistance marker, seeds were
germinated as described above. A single locus for the
trait was present in five out of nine transformants
with anther specific GUS expression that were tested.
30 The remaining transformants exhibited a ratio which was
greater than 3:1, indicating the presence of more than
one integration site.

     To establish lines of plants homozygous for the
TA29$_{500}$/P-GUS-NOS 3' gene 4-7 kanamycin resistant plants
35 from lines showing 3:1 segregation in the seed

germination test were potted in soil and grown to
maturity in the greenhouse. Seed was collected after
self fertilization, and seeds from the progeny of two
lines were germinated on kanamycin containing medium.

5 A population of seed that is 100% resistant was
identified for each line thereby establishing these as
homozygous populations.

EXAMPLE 5

The chimeric TA29$_{1500}$/P-GUS-NOS 3' gene was
10 introduced into tomato (_Lycopersicum esculentum_, var.
Herbs red cherry) by _Agrobacterium tumefaciens_
infection of cotyledon explants with the _Agrobacterium_
harboring pZ6ALG that was described above. Primary
transformants were analyzed to demonstrate anther
15 specific expression of this gene.

Standard aseptic techniques for the manipulation
of sterile media and axenic plant/bacterial cultures
were followed, including the use of a laminar flow hood
for all transfers. Ten days prior to inoculation,
20 tomato seeds were surface sterilized in a solution of
10% Clorox® and 0.1% SDS, then rinsed 3 times with
sterile water. Seeds were planted in magenta boxes
containing OMS agar medium (Table 2) and germinated in
a culture room under mixed fluorescent and grow lights
25 with a 16 hour day length at 25°C.

A single colony of _Agrobacterium_ harboring pZ6ALG
was used to inoculate 3 mL of MinA (Table 1) broth and
it was grown at 25°C with shaking over two nights. The
culture was diluted to an OD$_{650}$ of 0.1 in a flask
30 containing 30 mL of MinA broth and grown with shaking
at 28°C to a density equal to an OD$_{650}$ of 0.3. Using a
sterile scalpel, cotyledons were excised from seedlings
and approximately 2 mm of tissue was removed from each
end. These explants were planted underside down at 10
35 per plate on CTM agar (Table 2) containing 75 µM

acetosyringone in dimethylsulfoxide (DMSO). Then, 5 mL
of the _Agrobacterium_ solution was added to each plate,
making sure to wet all of the explants, and then
removed. The plates were incubated in the culture room

5   for 2-3 days. Thereafter, 15 mL of OMS solution (Table
2) containing 500 mg/L cefotaxime was added to each
plate and incubated for 10-15 minutes. The explants
were then transferred to sterile filter paper to remove
excess liqiud, then to plates of CTM medium containing

10 500 mg/L cefotaxime and 50 mg/L kanamycin, and
incubated in the culture room for approximately 3
weeks. Explants were then transferred to fresh plates
of CTM medium with 0.1 mg/L zeatin, 500 mg/L cefotaxime
and 50 mg/L kanamycin and incubated in the growth room

15 for 2-3 weeks. Fully formed shoots were excised,
dipped in 1 mg/L indole butyric acid, and planted in
magenta boxes containing 85 mL OMS gelrite (Table 2)
with 500 mg/l cefotaxime for root formation.

    To test the shoots for stable transformation, the

20 ability of leaf tissue to reshoot on kanamycin was
assayed. A leaf was removed from each shoot, cut into
2-3 pieces, and partially embedded in TB agar medium
(Table 2) with and without 25 mg/L kanamycin. Three to
four weeks after the shoots were excised, the ones that

25 showed kanamycin resistance in both the rooting and leaf
shooting assays were planted in 8 inch pots in Metro-
Mix, covered with a plastic bag (for a week), and grown
in a growth chamber set at 24°C, 12 hour daylength.
After several weeks, the plants were transferred to the

30 greenhouse and grown to maturity. Expression of the
TA29$_{1500}$/P-GUS-NOS 3' gene was assayed using the X-Gluc
GUS enzyme assay described above. Immature buds were
excised, dissected into pieces, and incubated in the
X-Gluc solution overnight. Blue staining was observed

35 only in the anthers of buds from all 6 of the plants

tested. S eds were collected following self-
fertilization of each transformant. Single locus
homozygous lines were identified as described in Example
3.

5   TABLE 2

OMS Medium
Per liter: 1 pkg MS Minimal Organic Medium without
                      sucrose (Gibco)
                  3 g sucrose
10   1 ml of B5 Vitamins
                  3 mM MES, [2-(N-morpholino)ethane-
                      sulfonic acid]
                  pH 5.7
                  8 g agar (for gelrite: add 0.75 g MgCl
15   and   2 g gelrite instead of agar)
CTM Medium
Per liter: 1 pkg MS Minimal Organic Medium without
                      sucrose (Gibco)
                  3 g sucrose
20   1 ml B5 vitamins: 1mg/mL nicotinic acid,
                      10 mg/mL thiamine hydrochloride,
                      1mg/mL pyridoxine hydrochloride,
                      100 mg/mL M-inositol
                  3 mM MES
25   pH 5.7
                  7 g agar
                  autoclave and add 1 mL of 1 mg/mL zeatin
TB Medium
Per liter: 1 pkg MS medium without sucrose
30   1 g sucrose
                  1 ml of B5 Vitamins
                  1 ml of 1 mg/mL BAP

0.4 mL of 1 mg/mL indole acetic acid
                (IAA)
        pH 5.8
        8 g agar

5

EXAMPLE 6

The chimeric $TA29_{1500}$/P-GUS-NOS 3' gene was
introduced into Brassica napus (cv. Westar) by
Agrobacterium tumefaciens infection of hypocotyl

10   explants with the Agrobacterium harboring pZ6ALG that
was described above. Primary transformants were
analyzed to demonstrate anther specific expression of
this gene.

Standard aseptic techniques for the manipulation

15   of sterile media and axenic plant/bacterial cultures
were followed, including the use of a laminar flow hood
for all transfers. Brassica seeds were surface
sterilized in a solution of 10% Clorox® and 0.1% SDS,
then rinsed thoroughly with sterile water. Seeds were

20   planted in 190 mm crystallization dishes containing 30
mM calcium chloride, 1.5% agar, and placed in the dark
at 25°C for five days.

A single colony of Agrobacterium harboring pZ6ALG
was used to inoculate 3 mL of MinA (Table 1) broth and

25   it was grown at 28°C with shaking for 18-20 hours. The
culture was grown to an OD_{650} of 1.0-2.0. Then 22.5 mL
of bacterial dilution medium (1 package MS minimal
organic medium and 30 g sucrose per liter) containing
100 µM acetosyringone was placed in a sterile dish.

30   Using a sterile scalpel, the seedling hypocotyls were
cut into 1 cm segments and placed immediately into the
bacterial dilution medium. 2.5 mL of the Agrobacterium
culture was added and after a 30 minute incubation the
hypocotyl pieces were placed on plates containing co-

35   cultivation medium: BC-1 (Table 3) containing 100 µM

acetosyringone. These plates were incubated in dim
light for 3 days at 25°C. The hypocotyl pieces were
then transf rr d to plat s of BC-1 medium containing
200 mg/L carbenicillin and 50 mg/L kanamycin and
5   incubated in the culture room. After 3, 6, and 9
weeks, calli growing from the cut hypocotyl ends were
transferred to plates of BS-48 shoot regeneration
medium (Table 3) containing 200 mg/L carbenicillin, and
incubated in continuous light at 25°C. The explants
10   were transferred to fresh medium of the same
composition every 2 weeks. Shoot primordia appeared
4-6 weeks after transfer to BS-48 medium and after
elongating they were transferred to MSV-1A medium
(Table 3) and incubated with a 10-12 hour photoperiod.
15   After 3 weeks each shoot tip along with several
internodes was transferred to fresh MSV-1A medium and
incubated for another 3 weeks. The shoots were then
cut near the agar surface, dusted with Rootone to
induce root formation, planted in 8 inch pots in Metro-
20   Mix, covered with a plastic bag (for 2 weeks), and
grown in a growth chamber set at 24°C, 16 hour
daylength. After several weeks, the plants were
transferred to the greenhouse and grown to maturity.
        Expression of the TA29$_{1500}$/P-GUS-NOS 3' gene was
25   assayed using the X-Gluc GUS enzyme assay described
above. Immature buds were excised, cut into pieces, and
incubated in the X-Gluc solution overnight. Blue
staining was observed in the anthers and not in other
parts of buds from all 4 of the plants tested. Seeds
30   were collected following self-fertilization of each
transformant.

35

TABLE 3

BC-1

Per liter: 1 pkg MS Minimal Organic Medium (Gibco)

                    30 g Sucrose

5   18 g Mannitol

                    0.2 mg 2,4-D

                    3 mg Kinetin

                    6 g DNA-Grade Agarose

                    pH 5.8

10   BS-48

Per liter: MS Minimal Organic Medium

                    1 mL B5 Vitamins: 1 mg/mL nicotinic

                            acid, 10 mg/mL thiamine

                            hydrochloride, 1mg/mL pyridoxine

15   hydrochloride, 100 mg/mL M-inositol

                    250 mg Xylose

                    10 g Glucose

                    0.6 g MES

                    4 g DNA Grade Agarose

20   pH 5.7

                    2 mg Zeatin (Add after autoclaving)

                    0.1 mg IAA (Add after autoclaving)

     MSV-1A

Per liter: MS Minimal Organic Medium

25   10 g Sucrose

                    1 mL B5 Vitamins

                    6 g DNA-Grade Agarose

                    pH 5.8

30   EXAMPLE 7

·A.   Isolation and Characterization of Anther-Specific
        cDNA from Brassica

        To obtain promoters from Brassica that are capable

of directing GUS expression specifically in anthers,

35   anther-specific cDNAs were isolated and characterized.

A cDNA library of <u>Brassica napus</u> (cv. Westar) was
constructed using poly(A)$^+$-RNA isolated from developing
anthers dissected from 2-3 mm flower buds. Total and
poly(A)$^+$-RNAs were purified using RNA Extraction and

5   mRNA Purification Kits (Pharmacia) according to the
manufacturer's specifications. cDNA was synthesized
using the RiboClone™ cDNA Synthesis System (Promega),
ligated with EcoRl adaptors (Promega), inserted into
the EcoRl site of the Lambda ZAP II vector

10   (Stratagene), and packaged into Lambda particles using
the GIGAPACK™GOLD Lambda DNA Packaging Extracts
(Strategene). This library was used for differential
screening using the standard phage plaque-lift method
(Bio-Rad). The probes were ^{32}P-labeled cDNA generated

15   both from (1) poly(A)$^+$-RNA purified from developing
anthers and (2) poly(A)$^+$-RNA purified from seedlings.
The putative positive Lambda ZAP II clones were
prepared to rescue the encompassed pBluescript plasmid
according to the supplier's instructions (Stratagene).

20   Six anther specific cDNA clones [p73 (SEQ ID NO:2),
p112 (SEQ ID NO:3), p54 (SEQ ID NO:4), p42W (SEQ ID
NO:5), p42 (SEQ ID NO:6) and p158 (SEQ ID NO:7)] in the
pBluescript vector (Stratagene) were identified by
Southern and Northern blots (Maniatis et al. 1982).

25   To verify the anther-specificity of the isolated
cDNAs and further characterize their expression, RNA
blots [Maniatis et al., <u>Molecular Cloning: A Laboratory</u>
<u>Manual</u>, Cold Spring Harbor Laboratory, New York (1982)]
containing total RNA purified from seedlings, petals,

30   filaments, different stages of anthers and pistils were
hybridized with the ^{32}P-labeled cDNA insert prepared
from each clone. The mRNAs encoded by p112 and p158
accumulated very early in the development of anthers,
in flower buds less than 2 mm in length, and the levels

35   of message declined and disappeared during anther

maturation. In contrast, the levels of mRNA encoded by
p54 and p73 cDNAs increased during anther development
and reached the peak level at anthesis. No
hybridization signal was observed in seedling, petal,
5   filament, and pistil RNAs. The expression of p42 and
p42W also showed anther specificity by Northern blot
analysis.

The techniques of in situ hybridization were used
to determine the tissue localization of mRNAs
10   represented by the cloned cDNAs in anthers of Brassica.
The techniques used are described by Cox and Goldberg
(1988, in Plant Molecular Biology: A Practical
Approach, C. H. Shaw, ed. Oxford: IRL Press, pp. 1-34).

Tissue sections were prepared from Brassica napus
15   anthers dissected from different stages of flower buds.
Sense and antisense probes were prepared from the cDNA
clones using the polymerase chain reaction (GeneAmp
kit, Perkin-Elmer Cetus) with universal and reverse
primers (Pharmacia), followed by preparation of
20   ^{35}S-labeled transcripts synthesized by T3 and T7 RNA
polymerase using methods suggested by the supplier of
the pBluescript vector (Stratagene). The p112
antisense probe hybridized only to the tapetal cell
layer surrounding the developing pollen grains and not
25   to any other cells in the section indicating that the
p112 mRNA is tapetal-cell specific. The control p112
sense probe did not show any specific hybridization.
Hybridization of the p158 antisense probe also showed
the p158 mRNA to be tapetum-specific, but the signal
30   level was lower than that of p112. These results were
consistent with the results of the Northern blots in
which the messages of p112 and p158 accumulated at
early stages of anther development and disappeared when
the tapetum layer was degenerated. Hybridization of
35   the p54 antisense probe showed pollen-specific

expression of p54 mRNA, which accumulated to a maximum
level in mature poll n. The _in situ_ hybridization
result for p42 show d that the mRNA was localized in
the outer parenchymatic cells of the vascular bundle,
5   the tapetal cell layer and pollen grains, but not in
the connective tissue or the outer cells of the
exotecium. The _in situ_ localization of p73 mRNA was
not examined, but its message accumulated at the
maximum level in the mature anther indicating it could
10 be another pollen-specific gene.

B.   Determination of DNA sequence of anther-specific
      cDNA clones

      DNA from cDNA clones p42, p42W, p54, p73, p112 and
p158 was made for sequence analysis by purifying
15 double-stranded plasmid using standard procedures
[Maniatis et al, _Molecular Cloning: A Laboratory_
Manual, Cold Spring Harbor Laboratory, New York
(1982)]. The nucleotide sequences of the cDNAs were
determined by the dideoxy method using a ^{T7}Sequencing™
20 kit (Pharmacia). Adjacent regions of the clones were
sequenced by priming with synthetic oligonucleotides
designed from sequences obtained from previous gel
readings.

      The cDNA and deduced amino acid sequences of p42
25 showed significant homology with chalcone synthase from
mustard [Ehmann and Schaefer, _Plant Mol. Biol_. 11:869-
870 (1988)], parsley [Reimold et al. _EMBO J_. 2: 1801-
1805 (1983)] and soybean [Akada et al. _Nucleic Acids_
Res. 18:3398 and 5899 (1990)]. The cDNA sequences of
30 p42W and p112 share 98% identity, and p42W has 18 extra
nucleotides at the 5'-terminus and 399 extra
nucleotides at the 3'-terminus. The sequences of the
cDNA clones are listed as SEQ ID NOS:2-7 for p73, p112,
p54, p42W, p42 and p158, respectively, and the

characterization for the _Brassica napus_ anther-specific
cDNA clones is summarized in Tabl 4.

C.   _Isolation of Anther-Specific Genomic Clones and_
      Promoters

5   A _Brassica napus_ genomic library of genes
corresponding to the isolated and characterized cDNA
clones are screened, by anther-specific cDNA inserts
which are ^{32}P-labeled as probes, using the standard
phage plaque-lift method (Bio-Rad). After the

10   isolation of a genomic clone coding for the anther-
specific cDNA, primer extension experiments [Ausubel et
al.], _Current Protocols in Molecular Biology_, John
Wiley & Sons, New York (1987)] are performed to
determine the transcription start site. The anther-

15   specific promoter is then isolated as the DNA segment
located 5' to the transcription start site. In this
way, novel _Brassica napus_ tapetum and pollen specific
promoters are prepared and used to construct chimeric
genes for anther-specific expression of GUS. The

20   promoter fragment is placed adjacent to the GUS-NOS
3'construction in pTZALG, after removing the TA29
promoter fragment. The resulting chimeric genes: 42/P-
GUS-NOS 3', 42W/P-GUS-NOS 3', 54/P-GUS-NOS 3', 73/P-
GUS-NOS 3', 112/P-GUS-NOS 3', and/or 158/P-GUS-NOS3'

25   are each cloned into pZS96. The genes are then
introduced into tobacco, tomato, and/or _Brassica_ as
described previously, and GUS expression is analyzed in
transformants as described in Example 9.

30

35

TABLE 4

Brassica napus Anther-Specific cDNAs

Clone	cDNA (kb)	mRNA[a] (kb)	Gene[b] copy #	In situ Localization	Homology
1. p42	0.75	~0.8	2-3	Tapetum, Pollen Periphery of VB[d]	Chalcone Synthase
2. p42W	0.97	~0.8	2-3	--	--
3. p54	0.41	~1.5	4-8	Pollen	--
4. p73	0.34	~0.8	3-5	--	--
5. p112	0.55	~0.8	2-3	Tapetum	--
6. p158	0.91[c]	~0.8	1-2	Tapetum	--

[a]Size estimated on Northern blots

5   [b]Copy number determined on Southern blots of genomic DNA

[c]p158 cDNA insert size estimated on agarose gel

[d]VB = vascular bundle

EXAMPLE 8

10   Chimeric genes for expression of GUS specifically in pollen can be constructed by making use of other pollen specific promoters known to one skilled in the art. LAT52 is a gene from tomato that is expressed preferentially in the pollen. The identification,

15   cloning, and characterization of this gene is described by Twell et al., Mol. Gen. Genet. 217: 240-245 (1989). Genes that are expressed specifically in pollen have also been studied in Zea mays and Tradescantia paludosa by Stinson et al., Plant Physiol. 83: 442-447 (1987).

20   In Hanson et al., The Plant Cell 1: 173-179 (1989) the isolation of the pollen specific Zmc13 gene is mentioned. To express GUS in pollen, a fragment containing the LAT52 or Zmc13 promoter region is prepared from the clone of the gene and placed adjacent

25   to the GUS-NOS 3' construction in pTZALG, after removing the TA29 promoter fragment. The resulting

chimeric genes LAT52/P-GUS-NOS 3' and Zmc13/P-GUS-NOS
3' are each cloned into pZS96 creating pZ6TGN and
pZ6CGN, respectively.

                          EXAMPLE 9

5   The chimeric LAT52/P-GUS-NOS 3' and Zmc13/P-GUS-
NOS 3' genes, described above, are introduced into
tobacco by infection of leaf disks with _Agrobacterium_
tumefaciens harboring pZ6TGN and pZ6CGN, respectively.
The procedure is carried out as described in Example 3.

10 Kanamycin-resistant transformants are tested for pollen
specific expression of GUS by the enzyme assays
described in Examples 4 and 5. Pollen is collected
from mature anthers, incubated in X-Gluc solution, and
blue staining of the pollen is assessed

15 microscopically. Alternatively, a protein extract is
prepared from collected pollen and assayed using the
MUG substrate. Samples of ovule and leaf tissue are
also tested for GUS activity as described above to
determine the specificity of expression in the pollen.

20 Seed is collected from plants showing anther specific
GUS activity, and homozygous lines are established as
described in Example 3.

        The chimeric LAT52/P-GUS-NOS 3' and Zmc13/P-GUS-
NOS 3' genes are introduced into tomato by infection of

25 cotyledon explants with _Agrobacterium_ _tumefaciens_
harboring pZ6TGN and pZ6CGN, respectively. The
procedure is carried out as described in Example 5.
Kanamycin-resistant transformants are tested for pollen
specific expression of GUS by the enzyme assays

30 described in Examples 4 and 5. Pollen is collected
from mature anthers, incubated in X-Gluc solution, and
blue staining of the pollen is assessed
microscopically. Alternatively, a protein extract is
prepared from collected pollen and assayed using the

35 MUG substrate. Samples of ovule and leaf tissue are

45

also tested for GUS activity as described above to
determine th specificity of expression in th pollen.
Seed is collected from plants showing anther-specific
GUS activity, and homozygous lines are established as
5   described in Example 3.

       The chimeric LAT52/P-GUS-NOS 3' and Zmc13/P-GUS-
NOS 3' genes are introduced into Brassica by infection
of hypocotyl explants with Agrobacterium tumefaciens
harboring pZ6TGN and pZ6CGN, respectively. The
10 procedure is carried out as described in Example 6.
Kanamycin-resistant transformants are tested for
pollen-specific expression of GUS by the enzyme assays
described in Examples 4 and 5. Pollen is collected
from mature anthers, incubated in X-Gluc solution, and
15 blue staining of the pollen is assessed
microscopically. Alternatively, a protein extract is
prepared from collected pollen and assayed using the
MUG substrate. Samples of ovule and leaf tissue are
also tested for GUS activity as described above to
20 determine the specificity of expression in the pollen.
Seed is collected from plants showing anther-specific
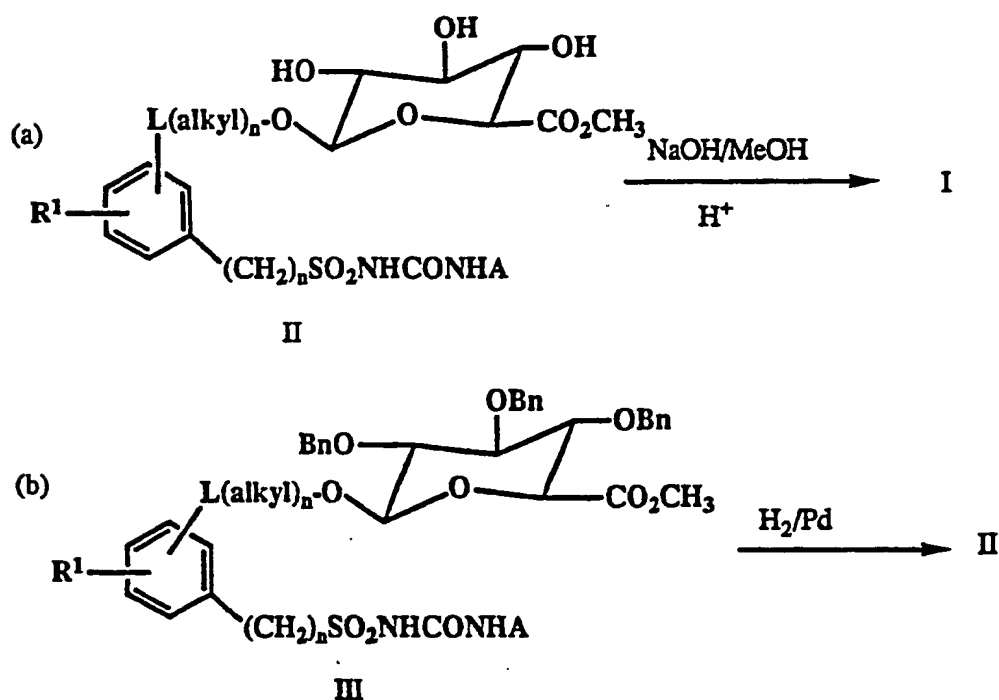GUS activity, and homozygous lines are established.

Preparation of the Protoxin
25   To form a β-O-glucuronide, the only structural
requirement on the aglycone is a hydroxyl or oxyanion
residue. As long as the residue is at least weakly
nucleophilic and is not severely sterically hindered,
conjugation can be performed by one or more of the
30 means outlined below. Examples of suitable substrates
include, but are not limited to, phenols, salts of
phenols, organic alcohols, salts of organic alcohols,
suitably activated carbonyl compounds such as 1,3-
dicarbonyls, imides, and activated secondary amides,
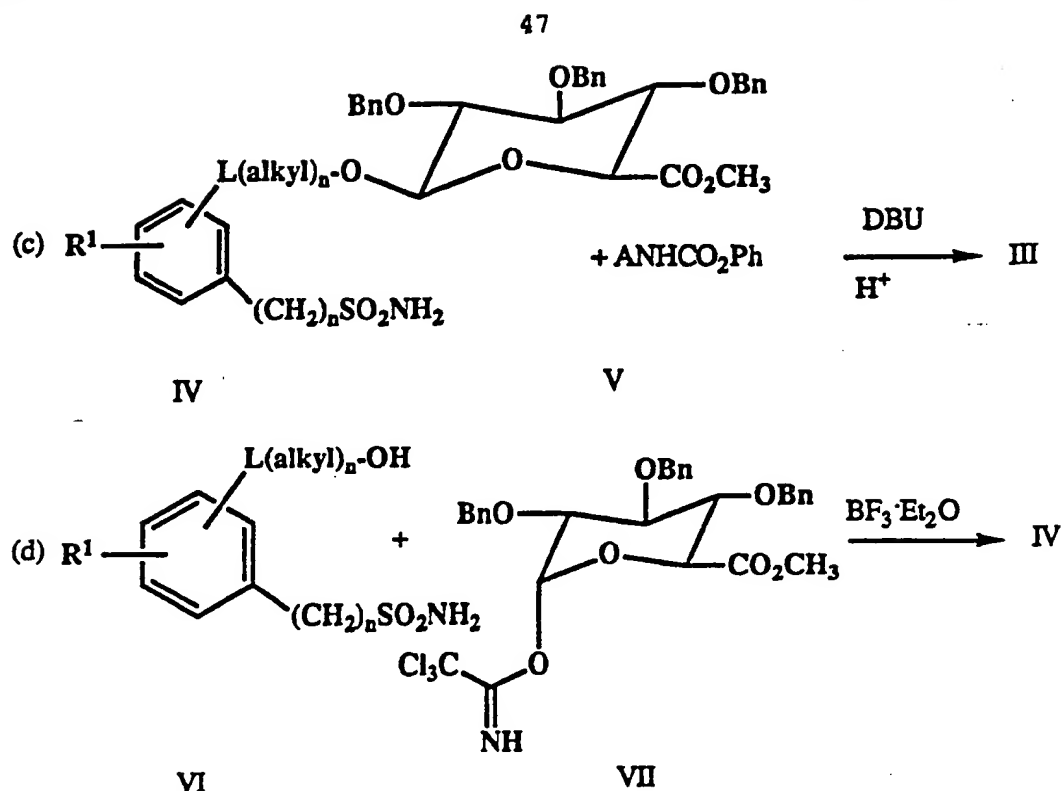35 and so forth. A variety of compounds that are

phytotoxic contain hydroxyl groups. Some of thes
compounds have been made into glucuronides and tested
on transgenic plants expressing male organ-specific
GUS.

5   The compounds of Formula I can be prepared by
either of two methods, differing by choice of
glucuronidating reagent. The first, Method A, utilizes
the reagent of Formula VII, whose synthesis is
described by Schmidt et al. in _Synthesis_, p. 885
10   (1981). Method A is outlined in Scheme 1 for compounds
of Formula I wherein J is J-1 but this method is
equally applicable to compounds wherein J is J-2 to
J-5.

15   _Scheme 1_

(a)

$L(alkyl)_n\text{-}O$

R^1

$(CH_2)_nSO_2NHCONHA$

HO, OH, OH, O, CO_2CH_3

$\xrightarrow[\text{H}^+]{\text{NaOH/MeOH}}$ I

II

(b)

$L(alkyl)_n\text{-}O$

R^1

$(CH_2)_nSO_2NHCONHA$

BnO, OBn, OBn, O, CO_2CH_3

$\xrightarrow{\text{H}_2/\text{Pd}}$ II

III

20

47

(c) R^1 —

BnO —, OBn, OBn, $L(alkyl)_n$-O, O, CO_2CH_3

$(CH_2)_nSO_2NH_2$

$+ ANHCO_2Ph$

$\xrightarrow[H^+]{DBU}$ III

IV   V

(d) R^1 —

$L(alkyl)_n$-OH

$(CH_2)_nSO_2NH_2$

$+$

BnO —, OBn, OBn, O, CO_2CH_3

Cl_3C O NH

$\xrightarrow{BF_3 \cdot Et_2O}$ IV

VI   VII

5   wherein:

n, R, R^1, and A are as previously described, and
Bn = CH_2Ph.

The reaction of Scheme 1(a) can be carried out by
10   contacting a glucuronide methyl ester of Formula II
with 2-5 equivalents of sodium hydroxide in methanol at
a temperature between 0 and 40°C for 0.1 to 24 hours.
The product can be isolated by adding a sufficient
amount of a cationic ion-exchange resin to protonate
15   the glucuronic acid sodium salt, filtering the resin,
and evaporating the solvent.
The reaction of Scheme 1(b) can be carried out by
the hydrogenolysis of a methanolic solution of a 2,3,4-
tri-O-benzyl glucuronide methyl ester of Formula III
20   with a palladium catalyst, such as 20% palladium
hydroxide on carbon (Pearlman's catalyst) at 0 to 30°C

for 0.1 to 24 hours under a hydrogen pressure of 1-10
atmospheres. The product can be isolated by filtration
and removal of solvent.

      The reaction of Scheme 1(c) can be carried out by
5   contacting a protected sulfonamide glucuronide of
Formula IV with an N-heterocyclyl carbamic acid phenyl
ester of Formula V, examples of which are well-known in
the art and which are known to form highly herbicidal
sulfonylureas with suitable sulfonamides. Generally,
10 equimolar quantitites of compounds of Formula IV and V
are dissolved in an inert solvent such as acetonitrile,
1 to 2 equivalents of an amidine base such as 1,8-
diazabicyclo[5.4.0]undec-7-ene (DBU) are added at -10
to 40°C, and the mixture is allowed to react for 0.1 to
15 2 hours, after which time the product is isolated by
acidification with aqueous acid and filtration or
extraction into a suitable organic solvent.
Purification can be accomplished by recrystallization
or chromatography on silica gel.
20   In the reaction of Scheme 1(d), the compound of
Formula IV can be prepared by the selective O-
glycosylation of a hydroxy-substituted sulfonamide with
the aforementioned reagent VII in the presence of an
acidic catalyst, such as boron trifluoride etherate in
25 a suitable solvent, such as dichloromethane, at -40 to
0°C for 0.1 to 2 hours. The product can be isolated by
washing the organic solution with aqueous $NaHCO_3$,
removing the solvent, and by chromatography on silica
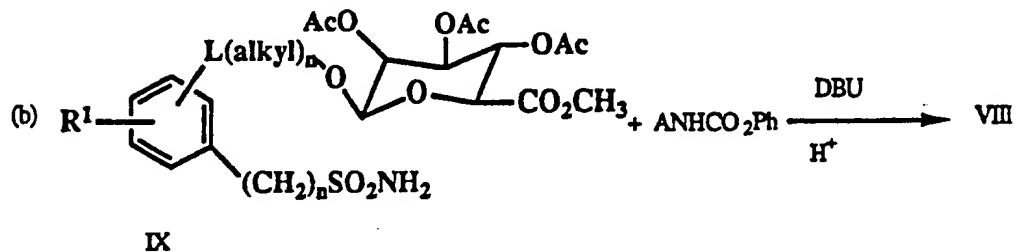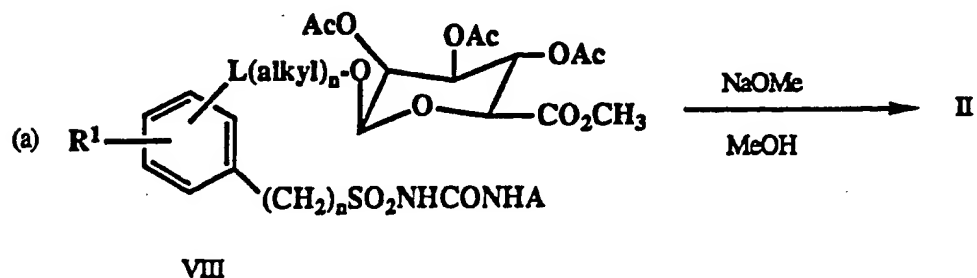gel and/or recrystallization.
30   The preparation of hydroxylated sulfonamides can
be accomplished by a wide variety of methods well-known
to one skilled in the art.

      An alternative procedure, Method B, utilizes a
commercially-available glucosylating reagent,
35 acetobromo-α-D-glucuronic acid methyl ester (XII), and
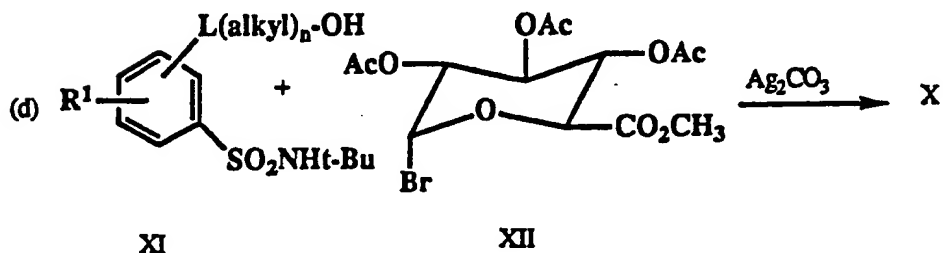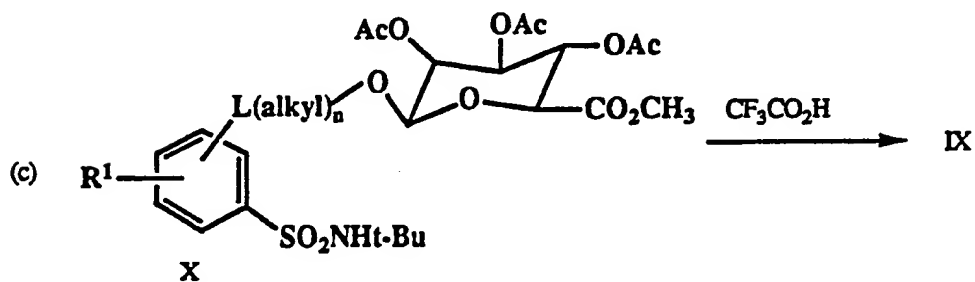
49

is outlined in Scheme 2 for the preparation of
compounds of Formula I wherein J is J-1. This method
works qually well for compounds of F rmula I wherein J
is J-2 to J-5.

5

Scheme 2

(a) Reaction of VIII with NaOMe / MeOH → II

(b) Reaction of IX + ANHCO$_2$Ph, DBU / H$^+$ → VIII

(c) Reaction of X with CF$_3$CO$_2$H → IX

(d) Reaction of XI + XII, Ag$_2$CO$_3$ → X

10

15

wherein:

    n, R, R^1, are as previously defined and Ac is
        acetyl.

5   The reaction of Scheme 2(a) can be carried out by
contacting a solution of a compound of Formula VIII in
methanol with a catalytic amount of sodium methoxide
(0.01 to 0.1 equivalents) for 0.1 to 10 hours at 0 to
30°C. The product can be isolated by neutralization of
10   the catalyst and concentration of the reaction mixture
and may optionally be purified by chromatography.

      The reaction of Scheme 2(b) can be carried out
exactly as described above for the reaction of Scheme
1(c). The reaction of Scheme 2(c) can be carried out
15   by contacting a compound of Formula X with
trifluoroacetic acid which can be used as a solvent at
0-40°C for 0.1 to 10 hours. The product of Formula IX
can be isolated by removal of excess trifluoroacetic
acid under vacuum and chromatography on silica gel.

20   The reaction of Scheme 2(d), can be accomplished
by contacting a hydroxylated t-butyl sulfonamide of
Formula XI with 1-2 equivalents of methyl 2,3,4-
triacetyl-1-bromo-α-D-glucuronate and 1-3 equivalents
of a silver salt such as silver carbonate in an inert
25   anhydrous solvent such as benzene or dichloromethane
for 1-100 hours. The product can be isolated by
filtration followed by chromatography of the filtrate
on silica gel.

      The preparation of t-butyl sulfonamides containing
30   hydroxylated side-chains can be accomplished by a
variety of methods well-known to one skilled in the
art.

      A third glucosylating method, Method C, can be
employed utilizing the commercially-available reagent

acetobromo-α-O-glucuronic acid, methyl ester (XII) and
is outlined in Scheme 3.

    The reaction of Schem 3(a) can be carried out as
described above for the reaction of Scheme 2(a). The

5   reaction of Scheme 3(b) can be carried out by
contacting XIV with a chemcial containing a hydroxyl
group, in the presence of 1-2 equivalents of N-
iodosuccinimide (NIS) and 0.5-1.5 equivalents of a
strong acid such as trifluoromethanesulfonic acid under

10   an inert atmosphere such as nitrogen, and in an inert
anhydrous solvent such as dichloromethane for 0.1-10 h
(Konradsson, et al; Tetrahedron Letter, Vol. 31 (30),
4313 (1990)). The product could be isolated by washing
the organic solution with aqueous $NaHCO_3$ and aqueous

15    $Na_2S_2O_3$ solution, removing the solvent, and by
chromatography on silica gel.

    The reaction of Scheme 3(c) can be carried out by
contacting XII with 1-10 equivalents of 4-penten-1-ol
in the presence of 1-2 equivalents of an inorganic

20   metal salt such as silver carbonate, at 0 to 40°C in an
anhydrous inert solvent such as benzene for 1-40 h.
The product is obtained by filtering the solid,
removing the solvent and by recrystallization or
chromatography on silica gel.

25

Scheme 3

(a)

XIII

52

(b)

XIV

XIII

(c)

XII

XIV
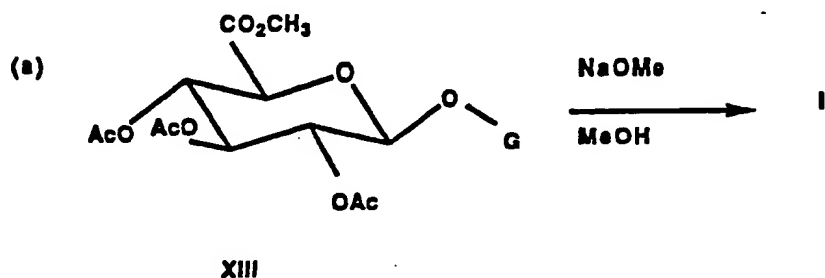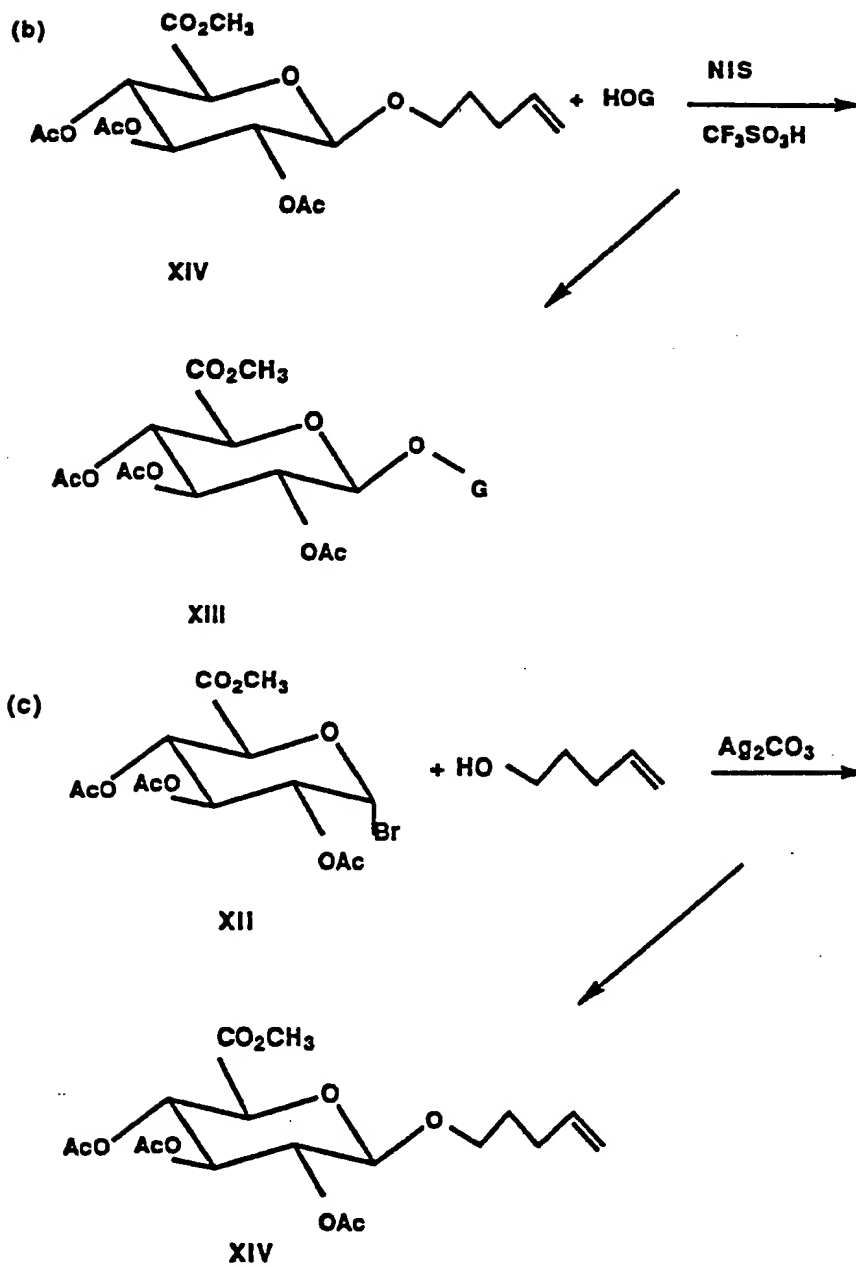
5   wherein G represents an aglycone toxin.

Another method to prepare the protoxins of the
instant invention is described in Scheme 4.

The reaction of Scheme 4(a) can be carried out as describ d above for the reaction of Scheme 2(a). The reaction of Scheme 4(b) can be carried out by contacting XII with a chemical containing a hydroxyl
5   group in the presence of 1-5 equivalents of an inorganic metal salt such as mercuric cyanide at 0-140°C, in an inert anhydrous solvent such as dimethylformamide for 0.1-10 h. The product is obtained by partitioning the reaction mixture between
10   water and an organic solvent such as ethyl acetate, combining the organic extracts and removing the solvent. Purification is by silica gel chromatography.

<u>Scheme 4</u>

15

(a)

(b)
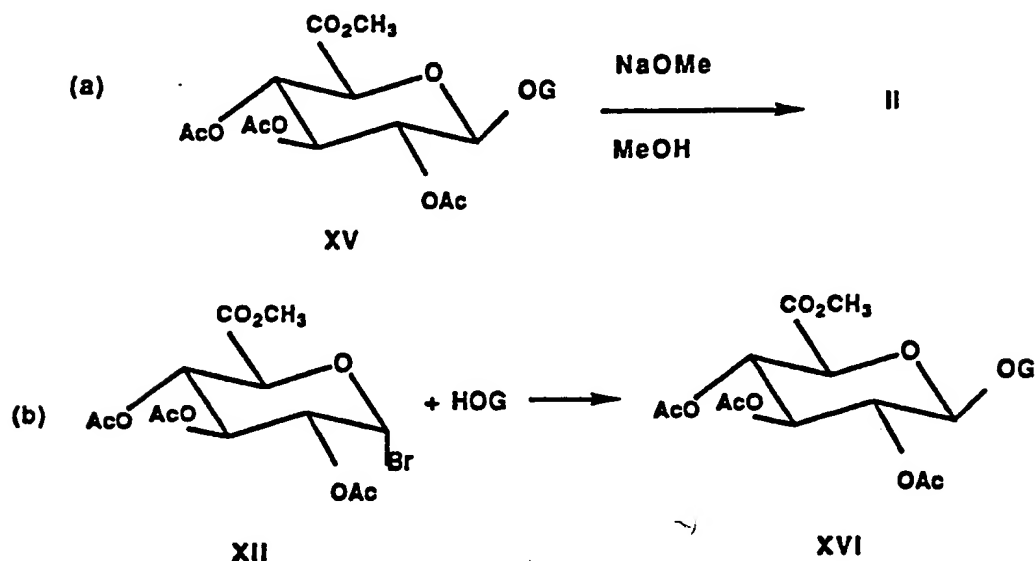
20   wherein G or HOG represent an aglycone toxin.

The following Examples illustrate the general methods outlined above.

<u>EXAMPLE 10</u>

[3-[2-[[[[(4,6-Dimethoxy-2-pyrimidinyl)-
amino]carbonyl]amino]sulfonyl]phenoxy]-
propyl]-β-L-gluco-pyranosiduronic acid

5   A.   A solution of 0.65 g of 2-(3-hydroxypropoxy)-
benzenesulfonamide and 2.2 g of compound VII in 70 mL
of dichloromethane was cooled to -25°C under a nitrogen
atmosphere and 5 drops of boron trifluoride etherate
was added, and the mixture was stirred for 1/2 hour at

10   -20°C.   Aqueous NaHCO3 was added, the CH2Cl2 layer was
separated, washed with brine, dried with MgSO4 ,
concentrated, and chromatographed on silica gel eluting
with an ethyl acetate-hexane mixture to provide 0.30 g
of the non-crystalline glucoside which was not further

15   purified.
B.   To a solution of 0.30 g of the glucoside from step
A in 5 mL of acetonitrile was added 0.10 g of phenyl N-
(4,6-dimethoxy-2-pyrimidinyl)carbamate, followed by
0.10 g of DBU.   The mixture was stirred at ambient

20   temperature for 10 minutes, and was then made acidic
with aqueous oxalic acid.   The product was extracted
with ethyl acetate, washed with brine, dried with MgSO4
and concentrated.   Application of the crude product to
a column of silica gel and elution with a mixture of

25   ethyl acetate and hexanes, and finally with pure ethyl
acetate afforded 0.3 g of the nearly pure sulfonylurea.
NMR (CDCl3) δ: 2.0 (multiplet, 2H), 3.4-4.2
(multiplet), 3.7 (singlet, 3H), 3.9 (singlet, 6H), 5.8
(singlet, 1H), 6.8 (doublet, 1H), 7.1 (triplet, 1H),

30   7.3 (multiplet), 7.5 (triplet, 1H), 8.2 (doublet of
doublet, 1H).   This was without additional purification
in step C.
C.   To a solution of 0.30 g of the product of step B
in 15 mL of methanol was added 0.15 g of 20% palladium

35   hydroxide-on-carbon (Pearlman's catalyst) and the

mixture was stirred under a hydrogen atmosphere for 2
hours at ambient temperature. The catalyst was removed
by filtration and the solution f the product was
concentrated and partially purified by chromatography
5   on silica gel, eluting with a gradient of methanol in
dichloromethane.

The intermediate methyl ester was saponified by
dissolving it in 10 ml of methanol and adding 1 mL of a
solution of 1N NaOH in 9 ml MeOH. After allowing the
10   solution to stand for 2 1/2 hours at ambient
temperature, it was stirred with excess ion-exchange
resin (acid-form of polystyrene-sulfonic acid resin),
filtered, and concentrated to provide 0.04 g of the
title compound. Fast-atom bombardment (FAB) mass
15   spectrum: 589 ($M^+ + 1$).

EXAMPLE 11

[2-[2-[[[[(4-Chloro-6-methoxy-pyrimidin-2-yl)-
amino]carbonyl]amino]sulfonyl]phenyl]ethyl]-
β-D-glucopyranosiduronic acid

20   A.   To methyl 2,3,4-triacetyl-1-bromo-α-D-glucuronate
(6 g, 15.11 mmole) in anhydrous benzene (400 mL) at
room temperature under N_2 atmosphere, N-(1,1-
dimethylethyl)-2-(2-hydroxyethyl)benzenesulfonamide
(4 g, 10.11 mmole) was added, followed by silver
25   carbonate (5 g, 18 mmole). The mixture was stirred at
room temperature for 24 hours. Another portion of
methyl 2,3,4-triacetyl-1-bromo-α-D-glucuronate (6 g,
15.11 mmole) and silver carbonate (5 g, 18 mmole) were
added. The mixture was further stirred for 24 hours
30   and was then suction filtered. The solid was washed
with benzene (100 mL). The filtrate and washings were
combined and concentrated under reduced pressure. The
residue was chromatographed on a silica gel column
(EtOAc/hexane = 1:1) to give the product as an oil
35   (5.5 g, 63% yield).

NMR (CDCl₃) δ: 1.23 (singlet, 9H), 1.95 (singlet, 3H),
2.04 (singlet, 6H), 3.10-3.63 (multiplet, 2H), 3.86
(singlet, 3H), 3.90-4.25 (multiplet, 3H), 4.68
(doublet, J = 7 Hz, 1H), 4.95-5.33 (multiplet, 3H),

5   7.28-7.57 (multiplet, 3H), 8.00-8.20 (multiplet, 1H).
B.   The product from step A (200 mg, 0.35 mmole) in
trifluoroacetic acid (5 mL) was stirred at room
temperature for 45 minutes. Trifluoroacetic acid was
removed under reduced pressure at room temperature.

10   The syrupy residue was redissolved in n-butylchloride
(10 ml) and concentrated under reduced pressure. This
was repeated once more to give a clear syrup which was
purified by silica gel column (eluted with ethyl
acetate) chromatography to give 0.160 gm (88%) of the

15   desired product as a colorless oil.
NMR (CDCl₃) δ: 1.70 (singlet, 3H), 1.98 (singlet, 3H),
2.00 (singlet, 3H), 3.35 (doublet of triplet, J_1 = 5 Hz,
J_2 = 15 Hz, 1H), 3.74 (singlet, 3H), 3.83 (multiplet,
1H), 4.05 (doublet, J = 10 Hz, 1H), 4.30 (multiplet,

20   1H), 4.48 (doublet, J = 8.5 Hz, 1H), 4.85 (triplet, J =
9.5 Hz, 1H), 5.00-5.15 (multiplet, 4H), 7.29-7.53
(multiplet, 3H), 8.05 (doublet, J = 7.5 Hz, 1H).
C.   To the product from step B (6.5 g, 12.29 mmole) in
acetonitrile (300 mL), the phenylcarbamate (3.44 g,

25   12.30 mmole) was added. The solution was cooled with
an ice bath and diazabicyclo[5.4.0]-undec-7-ene (2 mL)
was added. The reaction solution was stirred for 2
hours and was concentrated under reduced pressure. The
residue thus obtained was purified by silica gel column

30   chromatography (solvent system: MeOH/CH₂Cl₂ = 1:4) to
give the product as an oil (6.5 g, 74%).
NMR (DMSO) δ: 1.90 (singlet, 3H), 1.94 (singlet, 3H),
1.96 (singlet, 3H), 3.05-4.03 (multiplet, 4H), 3.62
(singlet, 3H), 3.70 (singlet, 3H), 4.02 (doublet, J = 6

35   Hz, 1H), 4.85-5.00 (multiplet, 3H), 5.17 (triplet, J =

7 Hz, 1H), 6.05 (singlet, 1H), 6.75 (doublet, J = 6 Hz,
1H), 7.00-7.20 (multiplet and a broad singlet, 2H),
7.30-7.50 (multiplet and a broad singlet, 2H), 7.83
(doublet, J = 5 Hz, 1H).

5   D.   The product from step C (0.1 g, 0.14 mmole) was
dissolved in methanol (10 ml). A catalytic amount of
NaOMe in MeOH was added. The reaction solution was
stirred at room temperature for 1 hour. A drop of
acetic acid was added. The solvent was removed under
10   reduced pressure at room temperature. The residue thus
obtained was purified on a silica gel column (eluted
with 10% MeOH in CH_2Cl_2) to give the desired product (a
foam, 74 mg, 90%).
NMR (DMSO) δ: 2.90-3.95 (multiplet, 7H), 3.59 (singlet,
15   3H), 3.78 (singlet, 3H), 4.08 (multiplet, 2H), 4.30
(doublet, 5 Hz, 1H), 5.03-5.10 (broad singlet, 1H),
5.12-5.20 (broad singlet, 1h), 5.23 (doublet, J = 4 Hz,
1h), 6.40 (singlet, 1H), 7.12-7.30 (multiplet, 3H),
7.91 (doublet, J = 5 Hz, 1H), 8.70 (broad singlet, 1H).

20   E.   The product from step D (70 mg, 0.1 mmole) was
dissolved in MeOH (2 mL), dioxane (1 mL) and water (1
mL). NaOH aqueous solution (1N, 1 mL) was added. The
mixture was stirred for 2 1/2 hours. The water was
evaporated with a stream of air to give the product as
25   an oil (70 mg).
NMR (CD_3OD) δ: 3.05-3.95 (multiplet, 7H), 3.00
(singlet, 3H), 4.45 (singlet, 6H), 4.65 (doublet, J =
3.5 Hz, 1H), 5.65 (doublet, J = 3.5 Hz, 1H), 6.20
(singlet, 1H), 6.90-7.20 (multiplet, 3H), 7.82
30   (doublet, J = 6 Hz, 1H).

Tables 5-12 contain representative protoxins that
can be made and used by procedures described
heretofore.

TABLE 5

REPRESENTATIVE PROTOXINS

Protoxin No.	R^1	p	$L(alkyl)_n$	X	Y	Z
1	H	0	$OCH_2CH_2CH_2$	OCH_3	OCH_3	CH
2	H	0	$OCH_2CH_2CH_2$	CH_3	OCH_3	CH
3	H	0	CH_2CH_2	Cl	OCH_3	CH
4	H	0	CH_2CH_2	CH_3	CH_3	CH
5	H	0	$OCH_2CH_2CH_2$	CH_3	OCH_3	N
6	H	0	$OCH_2CH_2CH_2$	OCH_3	OCH_3	N
7	H	0	OCH_2CH_2	CH_3	OCH_3	CH
8	H	0	OCH_2CH_2	CH_3	CH_3	CH
9	H	0	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
10	H	0	$CO_2CH_2CH_2$	CH_3	OCH_3	N
11	H	0	$CO_2CH_2CH_2CH_2$	OCH_3	CH_3	CH
12	H	0	$CO_2CH_2CH_2CH_2$	OCH_3	OCH_3	CH
13	H	0	$CH_2CH_2CH_2$	OCH_3	CH_3	N
14	H	0	$CH_2CH_2CH_2$	OCH_3	OCH_3	CH
15	H	0	$OCH(CH_3)CH_2$	Cl	OCH_3	CH
16	H	0	$OCH(CH_3)CH_2$	CH_3	OCH_3	N
17	6-F	0	$C(CH_3)_2CH_2CH_2$	CH_3	OCH_3	CH
18	6-F	0	$C(CH_3)_2CH_2CH_2$	CH_3	OCH_3	N
19	6-Cl	0	$SO_2CH_2CH_2CH_2$	OCH_3	OCH_3	CH
20	6-Cl	0	$SO_2CH_2CH_2CH_2$	OCH_3	CH_3	CH
21	5-CF_3	0	$CO_2CH_2CH_2$	CH_3	CH_3	CH
22	5-CF_3	0	$CO_2CH_2CH_2$	CH_3	OCH_3	CH

Protoxin No.	R^1	p	L(alkyl)$_n$	X	Y	Z
23	H	1	$SCH_2CH_2CH_2$	CH_3	CH_3	CH
24	H	1	$CH(CH_3)$	CH_3	OCH_3	CH
25	H	0	$OCH_2CH_2OCH_2CH_2$	OCH_3	CH_3	N
26	H	0	$C(CH_3)_2CH_2CH_2$	OCH_3	OCH_3	N
27	H	0	$CH_2C(CH_3)_2CH_2$	CH_3	OCH_3	N
28	H	0	CH_2	CH_3	CH_3	CH
29	H	0	$CH(CH_3)CH_2$	CH_3	OCH_3	CH
30	H	0	$CH_2SCH_2CH_2$	CH_3	CH_3	CH
31	H	0	$SO_3CH_2CH_2$	OCH_3	CH_3	N
32	H	0	$CH_2OCH_2CH_2$	OCH_3	CH_3	N
33	H	0	SCH_2CH_2	OCH_3	CH_3	CH
34	H	0	SCH_2CH_2	OCH_3	OCH_3	CH
35	H	0	$SO_2NHCH_2CH_2$	OCH_3	Cl	CH
36	6-Cl	0	$CH_2CH_2CH_2$	CH_3	CH_3	CH
37	6-Cl	0	$CH_2CH_2CH_2$	OCH_3	CH_3	CH
38	6-Cl	0	$CH_2CH_2CH_2$	OCH_3	OCH_3	CH

TABLE 6

REPRESENTATIVE PROTOXINS

Gluc-O-(alkyl)$_n$-L—[benzene ring positions 3,4,5,6,2 with R^1]—(CH$_2$)$_p$SO$_2$NHCONH—[triazine ring with X, Y, Z, N]
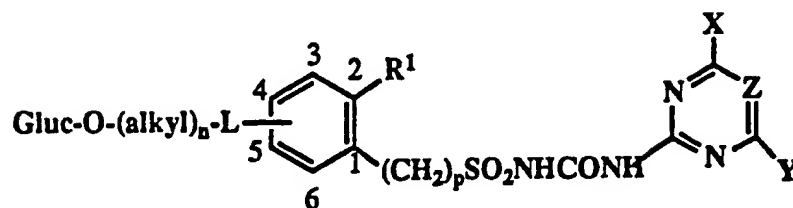
Protoxin No.	R^1	p	L(alkyl)$_n$	X	Y	Z
39	CO_2CH_3	0	5-CH_2	CH_3	CH_3	CH
40	CO_2CH_3	1	5-CH_2	OCH_3	CH_3	N
41	CO_2CH_3	0	3-OCH_2CH_2	CH_3	CH_3	CH
42	$CO_2CH_2CH_3$	0	3-CH_2	OCH_3	CH_3	N
43	$CO_2CH_2CH_3$	0	3-CH_2	OCH_3	CH_3	N

Protoxin No.	R^1	p	L(alkyl)$_n$	X	Y	Z
44	$CO_2CH_2CH_3$	0	5-$CH(CH_3)$	CH_3	OCH_3	CH
45	$CO_2CH_2CH_3$	0	3-CH_2CH_2	OCH_3	OCH_3	N
46	Cl	0	6-$CH_2CH_2CH_2$	CH_3	OCH_3	N
47	Cl	0	6-SCH_2CH_2	OCH_3	OCH_3	N
48	Cl	0	5-OCH_2CH_2	CH_3	CH_3	CH
49	Cl	0	6-$OCH_2CH_2CH_2$	OCH_3	CH_3	CH
50	$CH(CH_3)_2$	0	6-$SO_2CH_2CH_2$	CH_3	CH_3	CH
51	$CH(CH_3)_2$	0	6-CH_2	OCH_3	CH_3	CH
52	$CH(CH_3)_2$	0	5-$C(CH_3)_2CH_2CH_2$	OCH_3	OCH_3	N
53	SO_2CH_3	0	3-OCH_2CH_2	OCH_3	OCH_3	CH
54	SO_2CH_3	0	5-$OCH(CH_3)CH(CH_3)$	CH_3	OCH_3	CH
55	SO_2CH_3	0	6-$SO_2CH_2CH_2$	OCH_3	OCH_3	CH
56	$SO_2nC_3H_7$	0	6-OCH_2CH_2	OCH_3	CH_3	N
57	$SO_2nC_3H_7$	0	3-$OCH_2CH(CH_3)$	OCH_3	CH_3	CH
58	$SO_2nC_3H_7$	0	5-$CH(CH_3)CH_2$	CH_3	CH_3	CH
59	Br	0	5-$CO_2CH_2CH_2$	CH_3	CH_3	CH
60	CF_3	0	5-OCH_2CH_2	CH_3	OCH_3	CH
61	NO_2	0	5-CH_2	CH_3	OCH_3	N
62	NO_2	0	3-$OCH_2CH_2CH_2$	OCH_3	OCH_3	N
63	NO_2	0	6-OCH_2CH_2	OCH_3	OCH_3	CH
64	OCH_2CF_3	0	3-$OCH_2CH_2CH_2$	CH_3	CH_3	CH
65	$OCHF_2$	0	5-$C(CH_3)_2CH_2CH_2$	CH_3	CH_3	CH
66	SCF_2CF_2H	0	5-CH_2	CH_3	CH_3	CH
67	Ph	0	5-CH_2CH_2	OCH_3	OCH_3	N
68	$COCH_2CF_3$	0	5-$CH_2CH_2CH_2$	OCH_3	OCH_3	CH
69	$COCH_3$	0	5-$SO_2CH_2CH_2$	OCH_3	OCH_3	N

TABLE 7

REPRESENTATIVE PROTOXINS
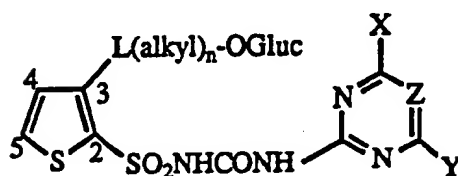
Protoxin No.	L(alkyl)$_n$	X	Y	Z
70	CH_2	CH_3	OCH_3	CH
71	CH_2	OCH_3	OCH_3	N
72	CH_2CH_2	CH_3	CH_3	CH
73	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
74	$CO_2CH_2CH_2$	CH_3	OCH_3	N
75	$CO_2CH_2CH_2CH_2$	OCH_3	OCH_3	CH
76	$SO_2CH_2CH_2$	CH_3	CH_3	CH
77	$SO_2CH_2CH_2$	CH_3	OCH_3	CH
78	$SO_2CH_2CH_2CH_2$	CH_3	CH_3	N
79	OCH_2CH_2	CH_3	OCH_3	CH

TABLE 8

REPRESENTATIVE PROTOXINS
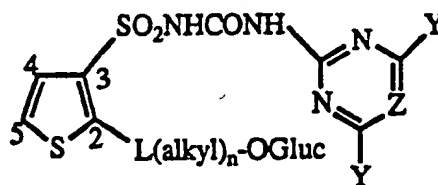
Protoxin No.	L(alkyl)$_n$	X	Y	Z
80	CH_2	CH_3	OCH_3	CH
81	CH_2	OCH_3	OCH_3	N

Protoxin No.	L(alkyl)$_n$	X	Y	Z
82	CH$_2$CH$_2$	CH$_3$	CH$_3$	CH
83	CO$_2$CH$_2$CH$_2$	CH$_3$	OCH$_3$	CH
84	CO$_2$CH$_2$CH$_2$	CH$_3$	OCH$_3$	N
85	CO$_2$CH$_2$CH$_2$CH$_2$	OCH$_3$	OCH$_3$	CH
86	SO$_2$CH$_2$CH$_2$	CH$_3$	CH$_3$	CH
87	SO$_2$CH$_2$CH$_2$	CH$_3$	OCH$_3$	CH
88	SO$_2$CH$_2$CH$_2$CH$_2$	CH$_3$	CH$_3$	N

TABLE 9

REPRESENTATIVE PROTOXINS
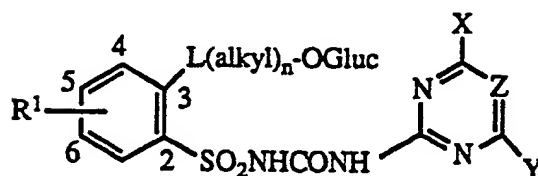
Protoxin No.	R^1	L(alkyl)$_n$	X	Y	Z
89	H	CO$_2$CH$_2$CH$_2$	CH$_3$	CH$_3$	CH
90	6-OCH$_3$	CO$_2$CH$_2$CH$_2$	CH$_3$	OCH$_3$	CH
91	6-CF$_3$	CO$_2$CH$_2$CH$_2$	CH$_3$	OCH$_3$	N
92	H	SO$_2$CH$_2$CH$_2$CH$_2$	OCH$_3$	OCH$_3$	CH
93	6-CH$_3$	SO$_2$CH$_2$CH$_2$CH$_2$	CH$_3$	OCH$_3$	N
94	6-CF$_3$	SO$_2$CH$_2$CH$_2$CH$_2$	OCH$_3$	CH$_3$	CH

63

TABLE 10

REPRESENTATIVE PROTOXINS

Protoxin No.	R^1	L(alkyl)$_n$	X	Y	Z
95	H	CH_2CH_2	CH_3	OCH_3	CH
96	H	$CH_2CH_2CH_2$	CH_3	OCH_3	N
97	H	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
98	$6-CF_3$	$SO_2CH_2CH_2CH_2$	CH_3	OCH_3	CH
99	H	SCH_2CH_2	OCH_3	OCH_3	CH
100	$6-Cl$	OCH_2CH_2	OCH_3	OCH_3	N

TABLE 11

REPRESENTATIVE PROTOXINS
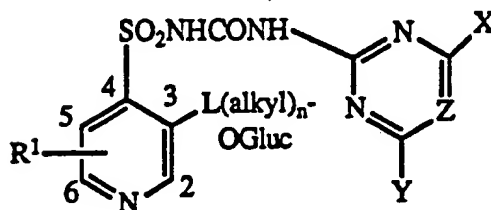
Protoxin No.	R^1	L(alkyl)$_n$	X	Y	Z
101	H	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
102	H	$CO_2CH_2CH_2$	OCH_3	OCH_3	CH

Protoxin No.	R¹	L(alkyl)$_n$	X	Y	Z
103	H	CH$_3$ N–CH$_2$CH$_2$	OCH$_3$	OCH$_3$	CH
104	H	CH$_3$ N–CH$_2$CH$_2$	OCH$_3$	CH$_3$	CH
105	H	CO$_2$CH$_2$CH$_2$	CH$_3$	OCH$_3$	N
106	H	CO$_2$CH$_2$CH$_2$CH$_2$	OCH$_3$	OCH$_3$	N
107	H	SO$_2$CH$_2$CH$_2$CH$_2$	CH$_3$	CH$_3$	CH
108	2-CF$_3$	SCH$_2$CH$_2$	OCH$_3$	CH$_3$	CH
109	6-Cl	CO$_2$CH$_2$CH$_2$	OCH$_3$	CH$_3$	CH
110	H	SO$_2$CH$_2$CH$_2$	OCH$_3$	CH$_3$	CH

TABLE 12

REPRESENTATIVE PROTOXINS
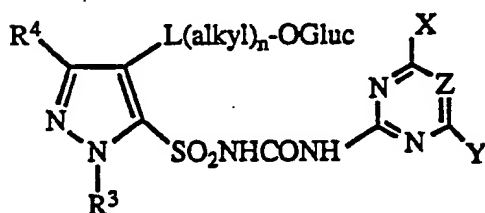
Protoxin No.	R³	R⁴	L(alkyl)$_n$	X	Y	Z
111	CH$_3$	H	CH$_2$CH$_2$	CH$_3$	OCH$_3$	CH
112	CH$_3$	H	CH$_2$CH$_2$	CH$_3$	OCH$_3$	N
113	CH$_3$	H	CH$_2$CH$_2$	CH$_3$	CH$_3$	CH
114	CH$_3$	H	CH$_2$CH$_2$	OCH$_3$	OCH$_3$	N
115	CH$_3$	H	CH$_2$CH$_2$	OCH$_3$	OCH$_3$	CH
116	CH$_3$	H	CO$_2$CH$_2$CH$_2$	CH$_3$	OCH$_3$	CH
117	CH$_3$	H	CO$_2$CH$_2$CH$_2$	CH$_3$	OCH$_3$	N
118	CH$_3$	H	CO$_2$CH$_2$CH$_2$	OCH$_3$	OCH$_3$	CH

EXAMPLE 12
1-(6-Hydroxy-3-pyradazinyl)-2,3,4-tri-_O_-acetyl-β-D-glucopyranosiduronic acid, methyl ester

A solution of maleic hydrazide (0.28 g, 2.5 mmole)

5   in anhydrous dimethylformamide (10 mL) under nitrogen
atmosphere and stirring, was warmed to 110°C. A solution
of methyl 2,3,4-tri-_O_-acetyl-1-bromo-α-D-glucuronate
(2 g, 5 mmole) in anhydrous dimethylformamide (6 mL) was
added in six portions, with a time interval of five

10 minutes between each addition. After the addition, the
reaction solution was further stirred at 110°C for 20
min. The reaction solution was then cooled to room
temperature, poured into water (100 mL), and extracted
with ethyl acetate (3 X 75 mL). The combined ethyl

15 acetate extracts were washed with brine, dried over
anhydrous magnesium sulfate, and filtered. The filtrate
was blown with a stream of air for 18 h. The residue
was taken up in methylene chloride. The undissolved
solid was filtered and the filtrate was concentrated

20 under reduced pressure to an oil which was
chromatographed on a silica gel column (EtOAc) to give
the title product as a yellow solid (0.36 g, 34% yield,
m.p. 162-165°C). Chemical Inonization (CI) mass
spectrum: 303 (M$^+$ + 1).

25   EXAMPLE 13
1-(6-Hydroxy-3-pyradazinyl)-β-D-glucopyranosiduronic acid, methyl ester

1-(6-Hydroxy-3-pyradazinyl)-2,3,4-tri-_O_-acetyl-β-D-
glucopyranosiduronic acid, methyl ester (2.0 g, 4.7

30 mmole) was dissolved in a mixture of methanol (40 mL)
and methylene chloride (20 mL) at room temperature. A
solution of sodium methoxide in methanol (25% by weight,
20 drops) was added. The reaction was monitored by thin
layer chromatography (methanol/methylene chloride =

35 1/4). When no more of the starting sugar was present,

resin (Amberlyst 15 ion exchange r sin, strongly acidic,
pre-washed with water until the washing was pH neutral)
was added to bring the pH of the reaction solution to
neutrality. The resin was then filtered, and the

5   filtrate was blown with a stream of air overnight to a
residue which was chromatographed on silica gel column
(methanol/methylene chloride = 1/4) to give the product
(0.5 gm, 35%, m.p. 180-183°C). Fast-atom bombardment
(FAB) mass spectrum: 303 (M^+ + 1).

10   EXAMPLE 14

                      Enzymatic Synthesis

       Some aglycones that are difficult to make into
glucuronides by conventional chemical synthesis means
can be glucuronidated by enzymatic procedures.

15   Glucuronidation is a major means of detoxification and
elimination of xenobiotics in mammals. The
glucuronidation enzyme, UDP-glucuronosyl transferase, is
present in many organs, with high levels in the liver.
The enzyme UDP-glucuronosyl transferase conjugates a

20   variety of aglycones onto UDP-glucuronic acid to form
β-O-glucuronides. Immobilized rabbit liver microsomal
UDP-glucuronosyl transferase has been shown to perform
conjugation in vitro. Some glucuronide protoxins were
prepared this way.

25   Liver microsomal UDP-glucuronosyl transferase was
extracted from phenobarbitol-treated New Zealand White
rabbits and immobilized as enzyme beads as described by
Lehman et al., Drug Metab. Disp., 9:15-18 (1981). For
most conjugation reactions, half of the reaction volume

30   was made of the suspended enzyme beads in 100 mM
phosphate buffer at pH 7.4. The concentrations of UDP-
glucuronic acid (ammonium salt) and the aglycone in the
reaction mix were 10 mM and 2 mM, respectively.
Magnesium chloride at 4 mM was used to facilitate the

35   reaction. If the aglycone was difficult to solubilize

in aqueous solution, up to 5% ethanol was used to
achi ve 2 mM concentration. Due to the relatively high
β-glucuronidase (GUS) activity in the crude rabbit liver
microsome enzyme preparation, 150 mM glucaro-1,4-lactone

5   was included in the reaction to suppress GUS activity.
If the reaction was run for more than one day, UDP-
glucuronic acid was replenished daily. The reaction
vessel was shaken gently at 37°C for the entire period.
        Progress of the enzyme conjugation reaction was

10 monitored by high pressure liquid chromatography (HPLC)
analysis of small aliquots of the reaction mixture. For
most analyses a C_{18} reverse phase column, 25 cm long, 4.6
mm inner diameter, was used. The mobile phase was a
mixture of water and acetonitrile (ACN) with 0.1% formic

15 acid. Usually the mobile phase gradient was from 5% ACN
to 80% ACN in 25 minutes, at a 1.4 mL per minute flow
rate. The column temperature was 35°C. After
sufficient glucuronide conjugate was synthesized, as
judged by the HPLC analysis, the reaction was

20 terminated. The enzyme beads were filtered out, and the
glucuronide was isolated from the reaction solution by
the following procedure: adjusted pH to 2.5/partition
3x with methylene chloride/ obtained a methylene
chloride phase (contains aglycone) and an aqueous

25 phase/partition 3x with n-butanol/obtain a butanol phase
(glucuronide) and an aqueous phase.
        The butanol fraction which contains the
glucuronide, a small amount of the aglycone and UDP-
glucuronic acid was evaporated to dryness in a rotary

30 evaporator. It was then further purified by a
preparative HPLC. A one-inch diameter C_{18} reverse phase
column, 25 cm in length, was used. An isocratic mobile
phase with 15 to 35% ACN in water, 0.1% formic acid was
used at a 15 mL per minute flow rate. The column was

35 kept at room temperature. The glucuronide peak was

collected by hand. Th pH of the glucuronide fraction
was quickly adjusted to neutrality. The ACN in the
fraction was evaporated by placing under a nitrogen gas
stream in a hood. The remaining aqueous fraction was

5   adjusted to pH 2.5 and extracted three times with
butanol. This butanol fraction was taken to dryness in
a rotary evaporator with gentle heating. The resulting
pure glucuronide was stored in a -20°C freezer.

Glucuronides of the following compounds (herbicidal

10 sulfonylureas with a free hydroxyl group) were prepared
by enzymatic techniques:

Cmpd.
No.

15   36   2-chloro-N-[[(4,6-dimethyl-2-pyrimidinyl)amino]-
carbonyl]-6-(3-hydroxypropyl)benzenesulfonamide,

37   2-chloro-6-(3-hydroxypropyl)-N-[[(4-methoxy-6-
methyl-2-pyrimidinyl)amino]carbonyl]benzene-
sulfonamide,

20   38   2-chloro-N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]-
carbonyl]-6-(3-hydroxypropyl)benzenesulfonamide,

119 2-[(2-hydroxyethyl)thio[-N-[[(4-methoxy-6-methyl-
2-pyrimidinyl)amino]carbonyl]benzenesulfonamide,

120 N-[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]-
25   carbonyl]-N'-(2-hydroxyethyl)-1,2-benzenedi-
sulfonamide,

121 N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-
6-(2-(N'-(2-hydroxyethyl)-N'-methyl)amino)-
2-pyridinesulfonamide,

30   122 6-[(2-hydroxyethyl)methylamino]-N-[[(4-methoxy-
6-methyl-2-pyrimidinyl)amino]carbonyl]-2-
pyridinesulfonamide, and

123 N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-
2-[(2-hydroxyethyl)thio]benzenesulfonamide.

The enzymatic conjugation procedures were as
described above. In all reactions, the glucuronides
showed a shorter retention time (about 2-2.5 minutes)
than their aglycones in the HPLC chromatogram. The UV
5   absorption spectra of all glucuronides were identical or
nearly identical to those of their respective aglycones.
When the aliquot of the reaction was incubated with the
GUS enzyme, the glucuronide peak disappeared and the
aglycone peak became larger. After purification,
10   glucuronides were again analyzed by HPLC to check sample
purity. The chemical identities of these glucuronides
were further confirmed by LC/MS/FAB analysis.

The substrate qualities of the glucuronides
produced were assayed by incubation with pure GUS
15   enzyme. The same concentration of glucuronide (0.5 mM)
was used in 2 mL, with 25 units (1 unit will liberate
1.0 µg of phenolphthalein from phenolphthalein
glucuronide per hour at 37°C) of GUS. At different time
points the reaction was stopped by pipetting in sodium
20   carbonate solution to raise the pH to 11.8. The
resulting samples were analyzed by HPLC. The peak area
ratio of the glucuronide over the aglycone was used to
indicate the degree of hydrolysis. It is clear that
different glucuronides were hydrolyzed by GUS at
25   different rates.

<div align="center">

EXAMPLE 15

Demonstration of in vivo β-glucuronidase activity in

TA29-GUS tobacco anthers

</div>

Although the GUS gene has been widely used in plant
30   molecular biology research, the demonstration of
β-glucuronidase enzyme activity is usually done in
vitro. The transgenic plant material harbouring the GUS
gene product, β-glucuronidase, is disrupted for color
staining or other assays for the enzyme activity. It is
35   generally assumed that the activity of β-glucuronidase

detected *in vitro* is well correlated with the *in vivo* activity.

To demonstrate β-glucuronidas activity *in vivo*, 4-methylumbelliferone glucuronide (MUG) was fed to

5 tobacco plants described in Examples 3 and 4, which exhibited anther-specific GUS expression by *in vitro* assays, using the cotton wick method as described below. Following different times after the feeding, anthers of young flowers were collected and assayed for the

10 presence of MUG, 4-methylumbelliferone (MU) and MU-glucoside. The phosphate buffer extract of anthers was divided into three aliquots. One aliquot was assayed for MU by fluorescence measurement. The second aliquot was treated with β-glucosidase, followed by MU

15 measurement. This value represents the MU, generated from MUG by the GUS enzyme, which was reconjugated into a beta-glucoside by the plant. The third aliquot was treated with the GUS enzyme, followed by MU measurement. This value represents the unhydrolyzed MUG in anthers.

20 The MU values of the first and second aliquots together represent the amount of MUG hydrolysis by GUS. It is clear that MUG, after being translocated into the anther, was abundantly hydrolyzed by the GUS enzyme into MU as described hereafter. Under similar conditions MUG

25 was minimally hydrolyzed in a non-transgenic plant.

EXAMPLE 16

Testing of protoxin/proCHA on transgenic plants producing male organ-specific exogenous enzyme

The protoxin solution was locally applied to the

30 inflorescence stalk of the transgenic plant through a fine cotton wick. The wick, pulled through the stalk by a sewing needle, was usually embedded at a point about one to two centimeters from flower clusters. The concentration of protoxin varied depending on the

35 toxicity of the aglycone and the size of the

inflor scence. One properly embedded wick can deliver
up to 1.5 mL of solution per day into the inflor scence
stalk, and it can function well for over a week.

Freshly shed pollen from treated flowers was
5   assayed for germinability in Brewbaker and Kwak medium
(15% sucrose, 300 ppm calcium nitrate, 100 ppm boric
acid). To test the degree of male sterility more
definitively, the same treated pollen was also used to
pollinate the stigma of untreated flowers. If a fruit
10   resulted from such pollination, it indicated that the
treatment did not cause complete male sterility.

Glucuronides of 2-[(2-hydroxyethyl)-thio]-N-[[(4-
methoxy-6-methyl-2-pyrimidinyl)amino]-carbonyl]-
benzenesulfonamide and N-[[(4,6-dimethoxy-2-
15   pyrimidinyl)amino]carbonyl]-6-[(2-hydroxyethyl)-
methylamino]-2-pyridinesulfonamide were fed into
infloresence stalks of tobacco plants described in
Examples 3 and 4 exhibiting anther-specific GUS activity
by the cotton wick method. At the beginning of the
20   feeding, the infloresence was very young with all of its
flower buds entirely enclosed within their sepals. The
concentrations of the glucuronides were between 1 - 3
parts per million (ppm), depending on the size of the
infloresence. The chemical solution uptake rate was
25   about 0.5 to 1.0 mL per day. More solution was added to
the small vial in which the cotton wick was bathed. The
solution addition was calibrated according to the growth
of flowers on the inflorescence. All treated
infloresences went on to produce flowers. Freshly shed
30   pollen from treated flowers was used to pollinate
untreated flowers. A small fraction of the pollen was
assayed for germinability in Brewbaker and Kwak medium
(supra). On the same day, treated flowers were
pollinated with pollen from untreated tobacco plants to
35   check their female fertility.

The female fertility of all treated flowers was
completely normal. A few days after receiving untreated
pollen, nice plump seed pods began to develop. However,
treated flowers failed to produce functional pollen.
5   Their freshly shed pollen grains showed no germination,
or much reduced germination, in Brewbaker and Kwak
medium. When such pollen was used to pollinate the
untreated flowers, much reduced rates of seed pod
formation were observed. The degree of male sterility
10 achieved by this localized chemical application via a
cotton wick can be variable. The outcome of the test is
influenced by a variety of factors, such as
environmental conditions, developmental stages of test
plant, position and size of the treated inflorescence.
15   EXAMPLE 17
Transgenic tobacco plants as described in Example 4
were sprayed with the methyl ester of maleic hydrazide
glucuronide. The pod-forming ability of said treated
plants was adversely affected versus control plants.
20

25

30

35

SEQUENCE LISTING

(1)   GENERAL INFORMATION:
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                         ODELL, JOAN T.

       (ii)   TITLE OF INVENTION: COMPOUNDS AND CONSTRUCTS FOR
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       (iii) NUMBER OF SEQUENCES: 8

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       (v)   COMPUTER READABLE FORM:

              (A)   MEDIUM TYPE: Floppy disk

              (B)   COMPUTER: IBM PC compatible

              (C)   OPERATING SYSTEM: PC-DOS/MS-DOS

              (D)   SOFTWARE: PatentIn Release #1.0, Version #1.25

       (vi)   CURRENT APPLICATION DATA:

              (A)   APPLICATION NUMBER:

              (B)   FILING DATE:

              (C)   CLASSIFICATION:

       (vii) PRIOR APPLICATION DATA:

              (A)   APPLICATION NUMBER: US 07/578,360

              (B)   FILING DATE: 06-SEP-1990

       (viii) ATTORNEY/AGENT INFORMATION:

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              (C)   TELEX: 835420

SUBSTITUTE SHEET

(2)   INFORMATION FOR SEQ ID NO:1:

    (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 549 base pairs

        (B)   TYPE: nucleic acid

        (C)   STRANDEDNESS: double

        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCTAGCTAA GTATAACTGG ATAATTTGCA TTAACAGATT GAATATAGTG CCAAACAAGA   60

AGGGACAATT GACTTGTCAC TTTATGAAAG ATGATTCAAA CATGATTTTT TATGTACTAA   120

TATATACATC CTACTCGAAT TAAAGCGACA TAGGCTCGAA GTATGCACAT TTAGCAATGT   180

AAATTAAATC AGTTTTTGAA TCAAGCTAAA AGCAGACTTG CATAAGGTGG GTGGCTGGAC   240

TAGAATAAAC ATCTTCTCTA GCACAGCTTC ATAATGTAAT TTCCATAACT GAAATCAGGG   300

TGAGACAAAA TTTTGGTACT TTTTCCTCAC ACTAAGTCCA TGTTTGCAAC AAATTAATAC   360

ATGAAACCTT AATGTTACCC TCAGATTAGC CTGCTACTCC CCATTTTCCT CGAAATGCTC   420

CAACAAAAGT TAGTTTTGCA AGTTGTTGTG TATGTCTTGT GCTCTATATA TGCCCTTGTG   480

GTGCAAGTGT AACAGTACAA CATCATCACT CAAATCAAAG TTTTTACTTA AAGAAATTAG   540

CTACCATGG   549

(2)   INFORMATION FOR SEQ ID NO:2:

    (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 337 base pairs

        (B)   TYPE: nucleic acid

        (C)   STRANDEDNESS: double

        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CACACACAAA AACTAAGTAA AAAGAAGAAA AAGCCTTTCA GGTCTCAACA ATGGAGATGA   60

AGAAGATCGC TTGCGGTGTG CTTTTTGTTG CTGCCTCCAT GACTGCTGTC ATGGCTATTG   120

AGGAAGCTGG AGCTCCGGCA CCAGGACCCG CATCCGCCGC CCTCGGTTGC ATTGCCGGCT   180

CTTGGCTCGT TGGTTGGTGC TTCGCTTGTG TCCCTATTCA GCTACTACTT GCACTAAGCT   240

ATGTCTTCAT TATGTAATAA TACTATTTCT GTGATCTTTT TCATTTCCTA CCTATTTCCA   300

TTTCACTTGT TTATTAATAA AACTGATAAA ATATTTC   337

(2)   INFORMATION FOR SEQ ID NO:3:
      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 554 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: double
            (D)   TOPOLOGY: linear
      (ii) MOLECULE TYPE: DNA (genomic)
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATCACATGCC TCCTCATCCT TACGATCTAC ATTGCAGCTC CAACAGAGTC ACACATAACG   60

TGTGGGACAG TGACAAGCAC AATGACACAG TGCATCAGCT ACTTGACCAA CGGTGGTCCA   120

TTGCCGTCAA GCTGCTGTGT GGCAGTCAAA TCATTGAACC AATGGCTCAG ACCCACCAGA   180

TCGCCGACAA GTAGTGTAGT CCTTAAATCA GCCGGTAAGG AAATTAAAGG CCTCAATATC   240

GACCTTGTGG CCGGCACTCC CTACCACTTG TGGTGTTTCA CTTTCATACC CCATTGGTTT   300

CAACACCAAT TGTGACAGTA TATCGATTGC CGTGTGAAGG AGACTAGAGA TGTACGAACG   360

AATAATCAAA GTTGGCCCGA CTTTAACCTA AACAATACTT CTGCTATTTT CTATTTTATG   420

ACTTGAGTTT CTTATTATGT GATCCATTTC ATATGGTAAT AAGTAATAAC GATAAAGGAG   480

CTGTCTCTCC TTTTCGACGC TTTGATGTAT GTACACGTAA CATACCATCA GGAATAACAC   540

TTTGTCTTTC TCTT   554

(2)   INFORMATION FOR SEQ ID NO:4:

    (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 406 base pairs

        (B)   TYPE: nucleic acid

        (C)   STRANDEDNESS: double

        (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGACAACCTT CAGGAATATT AGAGGAACAT CAGAGAACAA GGACGCAGTG AAACTATTGT   60

GCAGCAAGGG ACATCCATGT GAGAACGTTG AGATTGGAGA CATTAACATT GAGTACACAG   120

GACCTGACGG TCCACCCACT TTCGAGTGCA CAAACGTCAC ACCTAAGCTT GTGGGAGCCC   180

AGAACCCAAA GGCTTGCGTT GGACCTGTGG TCAAGGCTCC TGGCAAAGCG TAAAATGTTG   240

AAGCTCAGAT CAACAAACTA GGGCTTTCAC ATCCAATTTT GTTTTTCCTT TTCTCTAACC   300

CTTTTTCAAG TCACATATGG GTGAGTTTTA GAAAACTGTA ACCAAAAAAA TGTATATTCG   360

ATGCCAACAC ATATGTGTGA GTGCATAATG ATGTAATTAA TAAAAC   406

(2)   INFORMATION FOR SEQ ID NO:5:

    (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 971 base pairs

        (B)   TYPE: nucleic acid

        (C)   STRANDEDNESS: double

<pre>
            (D)   TOPOLOGY: linear
     (ii)   MOLECULE TYPE: DNA (genomic)
     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:5:
</pre>

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AGTGACAAGC ACAATGACAC AGTGCATCAG CTACTTGACC AACGGTAGGT CCATTGCCGT   60

CAAGCTGCTG TGTGGCAGTC AAATCATTGA ACCAAATGGC TCAGACCACA CCAGATCGCC  120

GACAAGTATG TGAGTGCCTT AAATCAGCCG GTAAGGAAAT TAAAGGCCTC AATATCGACC  180

TTGTGGCCGC ACTCCCTACC ACTTGTGTGT TTCACTTTCA TACCCCATTG GTTTCAACAC  240

CAATTGTGAC AGTATATCGA TTGCCGTGTG AAGGAGACTA GAGATGTACG AACGAATAAT  300

CAAAGTTGGC CCGACTTTAA CCTAAACAAT ACTTCTGCTA TTTCTATTTT ATGACTTGAG  360

TTTCTTATTA TGTGATCCAT TTCATATGGT AATAAGTAAT AACGATAAAG GAGCTGTCTC  420

TCCTTTTCGA CGCTTTGATG TATGTACACG TAACATACCA TCAGGAATAA CACTTTGTCA  480

TTTCCGTCTT ACAAATCCAA CAATTTATTA TAAACTAACT AAATAGACAT GATGAAGATC  540

TTGGCATTGA CACTCATGGT TTTCGTCATT CTTTCGCCAT CATTTGCGGC TCCAACTGAA  600

GTGGCACTCG GAGCGGCATG TGACGCTAAG CAGCTTCAGC CTTGCCTGGC AGCGATTACA  660

GGAGGAGGCC AACCCTCGGG TGATTGTTGT GCAAAGCTGA AGGAGCAGCA GCCATGCTTA  720

TGTGGATTTG CTAAGAACCC TGCGTTTGCT CAGTACATTA GCTCTCCGAA CTCTCGACAA  780

AGTCCTATCG CGTCTGATGC TGAGTCTATT CCTATCCAAT TGCGTAAAAC TTAGAGTTAT  840

AATTCAATAA ATAAATAAAT AACGTGTAAG GAAGATAAAT AACATTATAT ACTATGTTAA  900

CAAACAAGTA ACCGTTTGGT TAAGTAATCG TATACGGTTC ATCAAAGAAG TTTCTTATTT  960

AAAAAAAAAA A                                                      971
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(2)   INFORMATION FOR SEQ ID NO:6:

    (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 747 base pairs

        (B)   TYPE: nucleic acid

        (C)   STRANDEDNESS: double

        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:6:

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GGGATGCTAC GGAGGTGTGA GTGGGATGCG CGTGGCCAAG GACATTGCTG AGAACAACCC   60

AGGGAGCCGG GTGCTGCTCA CCACCTCCGA GACTATGGTT CTTGGGTTCC GTCCCCCCAA  120

CAAAGCTCGC CCTTACGACT TAGTCGGGGC TGCTCTCTTT GGAGACGGAG CAGCTGCCCT  180

GATCATCGGA GCAGACCCTA CAGAGTCAGA AAGTCCCTTC ATGGAGCTTC ACTATGCGCT  240

GCAGCAGTTC CTACCAGGAA CGCAGGCGGT GATTGATGGG CGGTTGTCTG AGGAGGGCAT  300

AAGCTTCAAG CTAGGAAGAG AACTACCTCA GAAGATCGAA GACAACATAG AGGAGTTCTG  360

CAAGAAGCTC GTGGCAAAGG CTGGCTCTGG TTCATTGGAG TTGAATGACC TGTTCTGGGC  420

CGTTCATCCC GGGTGGACCG CCATCCTGAA CGGGCTAGAG ACGAAACTGA AGCCTGAAAC  480

CAGAGAAGTT GGAATGCAGC AGACAGGCGT TGGTGGATTA TGGGAACGCA AGCAGCAACA  540

CCATCTTCTA CATAATGGAC AAAGTAAGAG ATGAGCTTGA GAAGAAAGGC AGAAGTGGAG  600

AAGAGTGGGG TCTGGGTTTA GCTTCGGACC GGGAATTACA TTCGAGGGAT TTCTCATGAG  660

GAGCCTCTAA ATGTGGCTAC TGGCTATGGA CCCGACATAT GTGATTAACT AGAAGTAAGA  720

ACAAATAAAT AAAACAGTCT CTTTTGC                                      747
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(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 871 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: double

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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GATTCTCGTG CCAGCAACTA TGCCACCACT TTGTTAACTA CGGGTTTCAC GACCTCCGGG   60

GGCTTAGGAA TGGTGGCTCT CCGCATCTTC TGGAAACTCT TCAAGTAATT AAATTAAACA  120

AAACCGTTAA TTGAAAGCCA CTCGAAAAAT GTGGTCTATT TATAATGACT GAGGCATTAT  180

GCATATGAAT CTACATGGAA ATATGTAAAT TTAATACATA TGCTTGGCTA TAATATAATT  240

ATTTACATCA ACGAAGATTC AGGTACATAG TTTCGTAGCT ACAATATATA NNNNNNNNCA  300

CATCTTCCGG CTTACCAAGT AATTAACAAA CCTGCGTTGA TGACACACTT AGATTAACTA  360

TTCTAAAACA CATTAGACGA TTAGTGTCGA GTGTTCAGGA GGCTGATTTC GCTATATTAG  420

TTTCTGAACA TAAAATAATT TATTTCTAAT TTTAAATATA TATCGTGTGT GCAGGCGTCT  480

TAGGAAGAAG GGGAAAGGAA CGCCAAAAAT TCCGGGATTG GCCCCGGGGG CTCCAGATTC  540

TGATCCAGTG TCTGGAGGAT AAACGGAGTA GGCTTTCGTA GTATGAGATA ATCTATGGAG  600

GAATTTAATG AGAATTGCTT GGGGTTCCTT AAATAAAACC TAAAATTATC CAGTGTCTTT  660

AACGTAAGAC TGATTACGCG CGGATGAAAC TAATGTTTCN NNNNNNNGGA TGAAACATTA  720

GTTATATACC ATTTTCAAAG AATATCGTGT TACGAATATA ATAAAGTACT CGATCATGTT  780

GTATATATTA AAGAATAAGT GATATCACTG AGTTACATAG ATGTATATTC AAGTTACATT  840
```

ATTGATTCAA TGAATTATTC GACTTTGAAA C   871

(2)   INFORMATION FOR SEQ ID NO:8:

    (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 30 base pairs

        (B)   TYPE: nucleic acid

        (C)   STRANDEDNESS: single

        (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGAAATTAGC TACCATGGTA GCTCCAAAAT   30

CLAIMS

What is claimed is:

5   1.   In a method for inducing sterility in the male
organ of a plant that comprises the steps:
        (i) transforming the plant with a DNA
construct that combines a male-organ specific promoter
with the coding region for β-glucuronidase; and
10   (ii) contacting the transformed plant with a
glucuronic acid-containing protoxin to release a toxin
which renders the male organ sterile;
the improvement that comprises:
        (a)   employing, in step (i), a promoter
15   selected from the group $TA29_{1500}$, $TA29_{500}$ (SEQ ID NO:1),
p73 (SEQ ID NO:2), p112 (SEQ ID NO:3), p54 (SEQ ID
NO:4), p42W (SEQ ID NO:5), p42 (SEQ ID NO:6) and p158
(SEQ ID NO:7); and
        (b)   employing, in step (ii), a protoxin that
20   comprises a toxin conjugated through a non-acyl, non-
phosphoryl hydroxyl residue to glucuronic acid.

     2.   A method according to Claim 1 comprising
transforming, in step (i), an agricultural or
25   horticultural plant.

     3.   A method according to Claim 2 wherein the
plant is selected from the group corn, rice,
orchardgrass, soybean, cotton, Brassica, pea, pepper,
30   potato, sugarbeet, alfalfa, sunflower, tobacco, flax,
tomato, lettuce, celery, carrot, eggplant, apple, melon,
petunia, periwinkle, poplar and walnut.

     4.   A DNA construct combining a promoter selected
35   from the group $TA29_{1500}$, $TA29_{500}$ (SEQ ID NO:1), and

promot rs from genes represent d by p73 (SEQ ID NO:2),
p112 (SEQ ID NO:3), p54 (SEQ ID NO:4), p42W (SEQ ID
NO:5), p42 (SEQ ID NO:6) and p158 (SEQ ID NO:7) with the
coding region for β-glucuronidase in the PZS96

5   Agrobacterium binary vector.

        5.   A protoxin comprising a sulfonylurea compound
or maleic hydrazide conjugated to glucuronic acid.

10   6.   A protoxin according to Claim 5 wherein the
conjugate is with a sulfonylurea compound.

        7.   A protoxin according to Claim 5 wherein the
cojugate is with maleic hydrazide.

15

        8.   A protoxin according to Claim 6 comprising a
sulfonylurea of Formula I including agriculturally
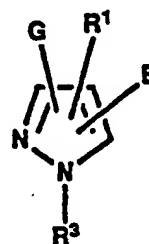suitable isomers, salts and derivatives thereof:

20
$$\begin{array}{c} W \\ \parallel \\ \mathrm{JSO_2NHCNA} \\ \mid \\ R \end{array}$$

25

                                 I

wherein J is

30   J-1   J-2   J-3
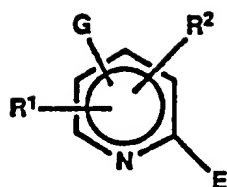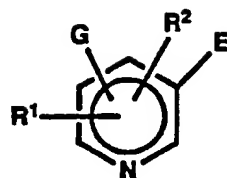
J-4   J-5

G is H or gluc-O(alkyl)$_n$L;

n is 0 or 1;

alkyl is 1 to 3 carbon atoms optionally substituted
    with one or two groups selected from halogen,
    methyl, methoxy or methylthio;

L is O; S(O)$_m$; NR^5; SO$_2$NR^4; CO$_2$; CH$_2$O; or a direct
    bond;

m is 0-3;

W is O or S;

R, R^4 and R^5 are independently H or CH$_3$;

E is a single bond or CH$_2$;

R^1 is H, C$_1$ to C$_3$ alkyl, C$_1$ to C$_3$ haloalkyl,
    halogen, nitro, C$_1$ to C$_3$ alkoxy, SO$_2$NR^{a}R^b,
    CONR^{a}R^b, C$_1$ to C$_3$ alkylthio, C$_1$ to C$_3$
    alkylsulfinyl, C$_1$ to C$_3$ alkylsulfonyl, CH$_2$CN, CN,
    CO$_2$R^c, C$_1$ to C$_3$ haloalkoxy, C$_1$ to C$_3$
    haloalkylthio, C$_2$ to C$_4$ alkoxyalkyl, C$_3$ to C$_4$
    alkoxyalkoxy, C$_2$ to C$_4$ alkylthioalkyl, CH$_2$N$_3$,
    NR^{d}R^e, or Q;

R^2 is H, C$_1$ to C$_3$ alkyl, C$_1$ to C$_3$ haloalkyl,
    halogen, nitro, C$_1$ to C$_3$ alkoxy, C$_1$ to C$_3$
    alkylthio, CN, C$_1$ to C$_3$ haloalkoxy, or C$_2$ to C$_4$
    alkoxyalkyl;

R^a is H, C$_1$ to C$_4$ alkyl, C$_2$ to C$_3$ cyanoalkyl,
    methoxy or ethoxy;

R^b is H, C$_1$ to C$_4$ alkyl or C$_3$ to C$_4$ alkenyl; or

R^a and R^b can be taken together as $-(CH_2)_3-$,
$-(CH_2)_4-$, $-(CH_2)_5-$ or $-CH_2CH_2OCH_2CH_2-$;

R^c is C_1 to C_4 alkyl, C_3 to C_4 alkenyl, C_3 to C_4
alkynyl, C_2 to C_4 haloalkyl, C_2 to C_3 cyanoalkyl,
C_5 to C_6 cycloalkyl, C_4 to C_7 cycloalkylalkyl or
C_2 to C_4 alkoxyalkyl;

R^d and R^e are independently H or C_1 to C_2 alkyl;

Q is a saturated or partially saturated 5- or
6-membered carbocyclic ring, containing either
one or two carbonyl groups, or a saturated or
unsaturated 5- or 6-membered heterocyclic ring,
containing 1 to 5 atoms of carbon and 1 to 4
heteroatoms selected from the group consisting
of 0 to 2 oxygen, 0 to 2 sulfur and 0 to 4
nitrogen, wherein sulfur can take the form of S,
SO or SO_2, and containing 0 to 2 carbonyl
groups; Q can further be optionally substituted
with 1 to 2 substituent groups; substituents on
carbon can be selected from the group consisting
of halogen, C_1 to C_4 alkyl, C_1 to C_4 haloalkyl,
$CH_2(C_2$ to C_3 alkenyl), $CH_2(C_2$ to C_3 alkynyl), C_2
to C_4 alkoxycarbonyl, CN, OH, C_1 to C_3 alkoxy, C_1
to C_3 alkylthio, C_1 to C_3 alkylsulfinyl, C_1 to C_3
alkylsulfonyl or C_2 to C_4 alkylcarbonyl;

substituents on nitrogen can be selected from
the group consisting of C_1 to C_4 alkyl, C_1 to C_4
haloalkyl, $CH_2(C_2$ to C_3 alkenyl), $CH_2(C_2$ to C_3
alkynyl), C_2 to C_4 alkoxycarbonyl or C_2 to C_4
alkylcarbonyl;

A is

;

X is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4
haloalkoxy, C_1 to C_4 haloalkyl, halogen, C_2 to C_5
alkoxyalkyl, C_2 to C_5 alkoxyalkoxy, amino, C_1 to
C_3 alkylamino, di(C_1 to C_3 alkyl)amino, C_3 to C_5
cycloalkyl, C_1 to C_4 alkyl substituted with
—O-gluc, C_2 to C_4 alkoxyalkyl substituted with
—O-gluc, or C_1 to C_4 alkoxy substituted with
—O-gluc;

Y is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4
haloalkoxy, C_2 to C_5 alkoxyalkyl, C_2 to C_5
alkoxyalkoxy, amino, C_1 to C_3 alkylamino or di(C_1
to C_3 alkyl)amino;

R^3 is H or C_1 to C_3 alkyl;

Z is CH or N;

E^1 is a direct bond or CH_2;

gluc is β-D-glucuronic acid;

provided that:

(i)   when G is H then X is C_1 to C_4 alkyl
      substituted with gluc, or C_1 to C_4 alkoxy
      substituted with gluc;

(ii) when L is not a direct bond, then n is not
      zero and alkyl comprises at least two carbon
      atoms which can be substituted as described.

          9.   A transgenic plant containing a DNA construct
     of Claim 4.

10.   A transgenic plant according to Claim 9 that
has been contacted with a protoxin comprising a
sulfonylur a compound or maleic hydrazide conjugated to
glucuronic acid.

5

11.   Hybrid seed from a plant according to any one
of Claim 9 or 10.

12.   A male-organ specific promoter, $TA29_{500}$ (SEQ
10   ID NO:1) comprising a nucleic acid fragment derived from
the TA29 gene that extends from the Eco RV restriction
site, which is about 500 base pairs 5' to the
transcription site and extending to the translation
initiation ATG.

15

13.   A male-organ specific promoter from genes
represented by p73 (SEQ ID NO:2), p112 (SEQ ID NO:3),
p54 (SEQ ID NO:4), p42W (SEQ ID NO:5), p42 (SEQ ID NO:6)
and p158 (SEQ ID NO:7).

20

14.   A method for planting crops preparatory to
field hybridization comprising the steps:
        (i)   planting crop A^1 and crop A^2 in
sufficient proximity that crop A^1 can be fertilized by
25   crop A^2;
        (ii)   inducing male sterility in A^1, before
fertilization, by a method according to Claim 1;
        (iii)   fertilizing A^1 with the pollen from A^2;
wherein A^1 comprises a plant lacking a desirable
30   heritable trait that is present in A^2 or will be present
in the hybrid progeny.

15.   A method according to Claim 14 comprising the
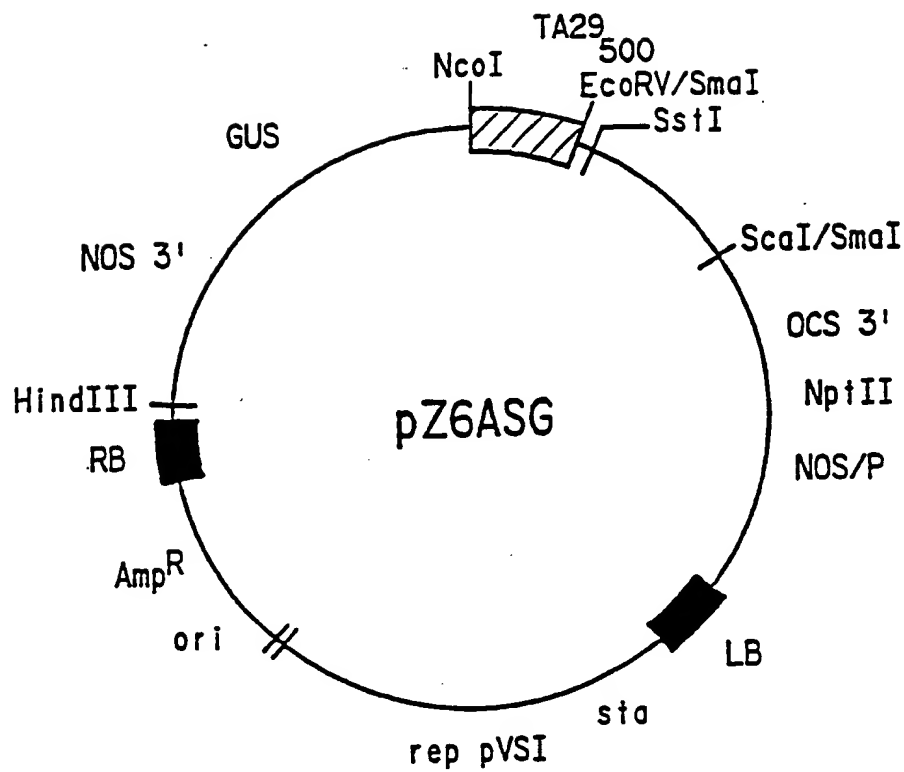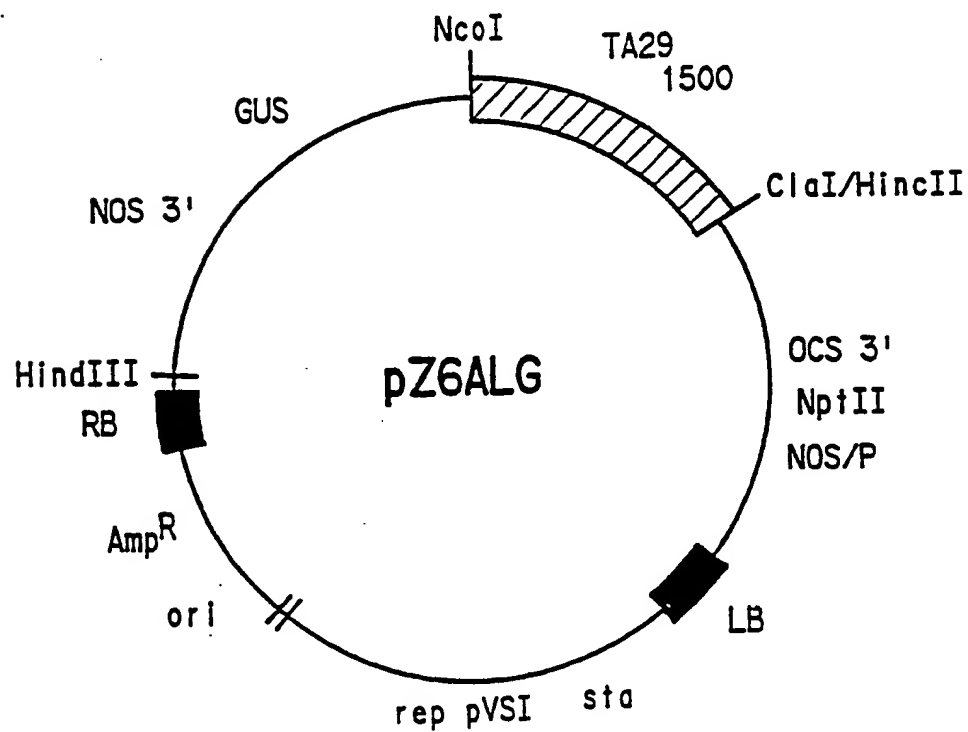additional step of harvesting hybrid seed from crop A^1.
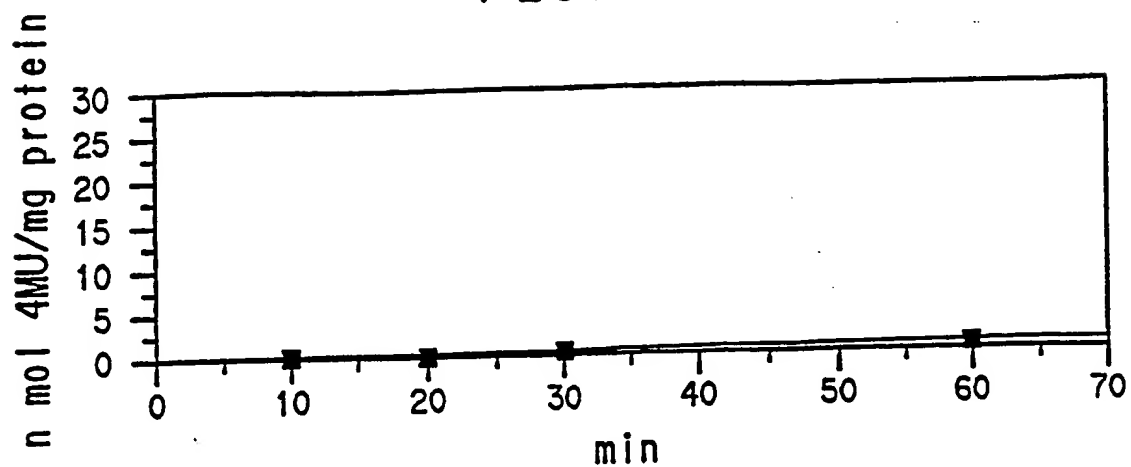
35

FIG.1A
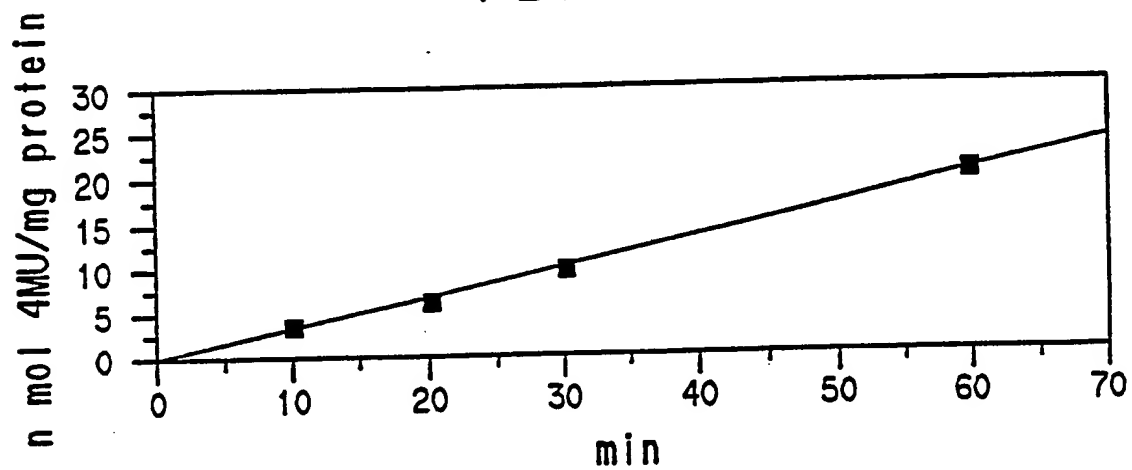

FIG.1B

FIG.2A

FIG.2B

FIG.2C

I. CLASSIFICATION OF SUBJECT MATTER   (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82;   C12N15/29;   C12N15/56;   C07H15/203
C07H15/26;   C07H17/02;   A01H1/02;   A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07H ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0 344 029 (PLANT GENETIC SYSTEMS) 29 November 1989	4,9
Y	see figure 5; examples 3,5,6	1-3,11, 14
Y	WO,A,9 008 828 (PALADIN HYBRIDS) 9 August 1990 see page 49, line 32 - page 50, line 3 see page 109, line 20 - line 35; figure 15	1-3,11, 14
Y	NATURE. vol. 342, 14 December 1989, LONDON GB pages 837 - 838; JEFFERSON, R. A.: 'The GUS reporter gene system' see page 838, left column	1-3,11, 14

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 JANUARY 1992	1 4 FEB 1992

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	CHEMICAL ABSTRACTS, vol. 103, 1985, Columbus, Ohio, US; abstract no. 16329, OIDA, T., ET AL: 'The metabolism of gliclazide in man' page 8 ; see abstract & XENOBIOTICA vol. 15, no. 1, 1985, pages 87 - 96; ---	5,6
X	CHEMICAL ABSTRACTS, vol. 88, 1978, Columbus, Ohio, US; abstract no. 31950, GAFITEANU, E., ET AL.: 'Elimination of p-bromohydrazino- and [p-bromophenyl]hydrazinopyridazine from the organism' page 21 ; see abstract & REV. MED.-CHIR. vol. 78, no. 3, 1974, pages 703 - 706; ---	5,7
P,X	THE PLANT CELL. vol. 2, no. 12, December 1990, ROCKVILLE, MD, USA. pages 1201 - 1224; KOLTUNOW, A. M.,ET AL.: 'Different temporal and spatial gene expression patterns occur during anther development' see figure 12 ---	4,12
A	EP,A,0 329 308 (PALADIN HYBRIDS) 23 August 1989 see the whole document ---	4,13
A	WO,A,9 008 830 (ICI) 9 August 1990 see examples 9,10 ---	4

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The members are as contained in the European Patent ffice EDP file on
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		WO-A-	8910396	02-11-89
		JP-T-	2503988	22-11-90
WO-A-9008828	09-08-90	AU-A-	5037290	24-08-90
		EP-A-	0456706	21-11-91
EP-A-0329308	23-08-89	AU-A-	2963289	03-08-89
WO-A-9008830	09-08-90	AU-A-	4945690	24-08-90
		EP-A-	0455665	13-11-91

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